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Patent- og Varemærkestyrelsen
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Pia Petersen

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PATENT- OG VAREMÆRKESTYRELSEN

ANTISENSE DESIGN

Field of Invention

The present invention relates to pharmaceuticals comprising antisense oligonucleotides, and novel oligonucleotides having improved antisense properties.

Background of the Invention

The Professors Imanishi and Wengel independently invented Locked Nucleic Acid (LNA) in 1997 (International Patent Applications WO 99/14226,WO 98/39352; P. Nielsen et al, *J. Chem. Soc., Perkin Trans. 1*, 1997, 3423; P. Nielsen et al., *Chem. Commun.*, 1997, 9, 825; N. K. Christensen et al., *J. Am. Chem. Soc.*, 1998, 120, 5458; A. A. Koshkin et al., *J. Org. Chem.*, 1998, 63, 2778; A. A Koshkin et al. *J. Am. Chem. Soc.* 1998, 120, 13252-53; Kumar et al. *Bioorg, & Med. Chem. Lett.*,1998, 8, 2219-2222; and S. Obika et al., *Bioorg. Med. Chem. Lett.*, 1999, 515). The first LNA monomer was based on the 2'-O-CH_2-4' bicyclic structure. Due to the configuration of this structure it is called: beta-D-oxy-LNA. This oxy-LNA has since then showed promising biological applications (Braasch & Corey, *Biochemistry*, 2002, 41(14), 4503-19; Childs et al. *PNAS*, 2002, 99(17), 11091-96; Crinelli et al., *Nucl. Acid. Res.*, 2002, 30(11), 2435-43; Elayadi et al., *Biochemistry*, 2002, in press; Jacobsen et al., *Nucl. Acid. Res.*, 2002, 30(19), in press; Kurreck et al., *Nucl. Acid. Res.*, 2002, 30(9), in press; Simeonov & Nikiforov, *Nucl. Acid. Res.*, 2002, 30(17); Alayadi & Corey; *Curr. opinion in Inves. Drugs.*, 2001, 2(4), 558-61; Obika et al., *Bioorg. & Med. Chem.*, 2001, 9, 1001-11; Braasch & Corey, *Chem. & Biol.*, 2000, 55, 1-7; Wahlestedt et al., *PNAS*, 2000, 97(10), 5633-38), Freier & Altmann, *Nucl. Acid Res.*, 1997, 25, 4429-43; Cook, 1999, *Nucisides & Nucleotides*, 18(6&7), 1141-62.

Right after the discovery of oxy-LNA the bicyclic furanosidic structure was chemically derivatised. Thus, the 2'-S-CH_2-4' (thio-LNA) and the 2'-NH-CH_2-4' (amino-LNA) bicyclic analogues were disclosed (Singh, S. K., *J. Org. Chem.*, 1998, 63, 6078-79; Kumar et al. *Bioorg, & Med. Chem. Lett.*,1998, 8, 2219-2222; Singh et al. *J. Org. Chem.*, 1998, 63, 10035-39). The synthesis of the thio-LNA containing uridine as nucleobase has been shown (Singh, S. K., *J. Org. Chem.*, 1998, 63, 6078-79). For amino-LNA the synthesis of the thymidine nucleobase has been disclosed (Kumar et al. *Bioorg, & Med. Chem. Lett.*,1998, 8, 2219-2222; Singh et al. *J. Org. Chem.*, 1998, 63, 10035-39). A series of LNA-diastereoisomers have been prepared (Rajwanshi et al., *J. Chem Commun.* 1999;2073-2074; Hakansson & Wengel, *Bioorg Med Chem Lett* 2001; 11(7):935-938; Rajwanshi et al. , *Chem Commun.*, 1999;1395-1396; Wengel at al., *Nucleosides Nucleotides Nucleic Acids*, 2001; 20(4-7):389-396; Rajwanshi et al., *Angew. Chem. Int. Ed.*, 2000; 39:1656-1659; Petersen et al., *J. Amer. Chem. Soc.*, 2001, 123(30), 7431-32;

Sørensen et al., *J. Amer. Chem. Soc.*, 2002, 124(10), 2164-76; Vester et al., *J. Amer. Chem. Soc.*, 2002, in press.). In the prior art the synthesis of alpha-L-xylo, xylo-LNA, and alpha-L-oxy-LNA containing thymidine bases have been shown. For the alpha-L-oxy-LNA also the 5-methyl and adenine nucleosides have been synthesised. The melting temperature (T_m) of duplexes containing the LNA distereoisomers have been presented. It turned out that the alpha-L-oxy-LNA has interesting properties. It was shown that the alpha-L-oxy-LNA can be incorporated in complex chimerae comprising DNA/RNA residues and be adapted in the oligo structure and increase the binding. This property of being incorporated in oligonucleotides containing several other monomeric classes and act co-operatively is a property that the alpha-L-oxy-LNA shares with the parent oxy-LNA. Furthermore it has been demonstrated that a segment of 4 consecutive alpha-L-T monomers can be incorporated in conjunction with a segment of 4 consecutive oxy-LNA-T monomers (Rajwanshi et al., *Chem. coummun.*, 1999, 2073-74). Increased stability of oligonucleotides containing alpha-L-oxy-LNA monomers (^{Me}C, A, T-monomers) have been demonstrated. The alpha-L-oxy-LNA monomers were incorporated into oligonucleotides with alternating alpha-L-oxy-LNA and DNA monomers (mix-mers) and in fully modified alpha-L-oxy-LNA oligomers. The stability was compared to oxy-LNA and to DNA and it was found that alpha-L-oxy-LNA monomers displaced the *same* protection pattern as oxy-LNA (Sørensen, et al., J.Amer.Chem.Soc., 2002, 124(10), 2164-76). The same alpha-L-oxy-LNA containing oligonucleotides were tested in RNase H assays and it was found that the designs disclosed were not efficiently recruiting RNase H. When these examples are taken together also in combination with the data published by Arzumanov et al (Biochemistry 2001, 40, 14645-54) it has not been shown that alpha-L-oxy-LNA containing oligonucleotides *efficiently* recruits RNase H.
Oligonucleotides containing any combination of the diastereoisomers and any other LNA family member has not been demonstrated.

Natural dsDNA exists at physiological pH as a B-form helix, whereas dsRNA exists as an A-form helix. A helix formed by DNA and RNA exists in an intermediate A/B-form. This morphological difference is originated in the difference in the preferred sugar conformations of the deoxyriboses and the riboses. The furanose ring of deoxyribose exists at room temperature in an equilibrium between C2'-*endo* (*S*-type) and C3'-*endo* (*N*-type) conformation with an energy barrier of ~2 kcal/mol (Figure 3). For deoxyribose the S-type conformation is slightly lowered in energy (~0.6 kcal/mol) compared to the N-type and explains why DNA is found in the S-type conformation. The conformation leads to the B-form helix. For ribose, and RNA, the preference is for the N-type that leads to the A-form helix. The A-form helix is associated with higher hybridisation stability. The oxy-LNA and the LNA analogues are locked in the N-conformation and consequently the oligonucleotides they are forming will be RNA-like. The alpha-L-oxy-LNA is locked in a S-type and therefore

the oligonucleotides that it will form will be more DNA like (Sørensen et al., *J. Amer. Chem. Soc.*, 2002, 124(10), 2164-76; Rajwanshi et al., Angew. Chem. Int. Ed., 2000; 39:1656-1659). Molecular strategies are being developed to modulate unwanted gene expression that either directly causes, participates in, or aggravates a disease state. One such strategy involves inhibiting gene expression with oligonucleotides complementary in sequence to the messenger RNA of a target gene. The messenger RNA strand is a copy of the coding DNA strand and is therefore, as the DNA strand, called the sense strand. Oligonucleotides that hybridise to the sense strand are called antisense oligonucleotides. Binding of these strands to mRNA interferes with the translation process and consequently with gene expression. Zamecnik and co-workers originally described the Antisense strategy and the principle has since then attracted a lot of interest (Zamecnik & Stephenson, PNAS, 1978, 75(1), 280-4; Bennet & Cowset, Biochim. Biophys. Acta, 1999, 1489, 19-30; Crooke, 1998, Biotechnol.Genet.Eng Rev., 15, 121-57; Wengel, J. In Antisense Drug Technology; Principles, Strategies, and Applications; Edited by Crooke, S. T., Ed.; Marcel Dekker, Inc.: New York, Basel, 2001; pp 339-357).

It has been a long sought goal to develop drugs with the capacity to destroy malignant genes base specifically. The applications of such drugs in e.g. cancer and infections diseases are self-evident. Native oligonucleotides cannot be employed as such mainly due to their instability in cellular media and to too low affinity for the target genes. The wish to develop nucleic acid probes with improved properties in this regard has been the main driver behind the massive synthesis effort in the area of nucleic acid analogue preparation. The most important guideline in this work has been to design the DNA analogues in such a way that the DNA analogue would attain the N-type/"RNA"-like conformation that is associated with the higher affinity of the oligonucleotides to nucleic acids.

One of the important mechanisms involved in Antisense is the RNase H mechanism. RNase H is an intra cellular enzyme that cleaves the RNA strand in RNA/DNA duplexes. Therefore, in the search for efficient Antisense oligonucleotides, it has been an important hallmark to prepare oligonucleotides that can activate RNase H. However, the prerequisite for an oligonucleotide in this regard is therefore that the oligo is DNA-like and as stated above most high affinity DNA analogues induces RNA-like oligonucleotides. Therefore, to compensate for the lack of RNase H substrate ability of most DNA analogues (like e.g. 2'-Ome DNA analogue and oxy-LNA) the oligonucleotides must have segments/consecutive stretches of DNA and/or phosphorothioates. Depending on the design of the segments of such oligonucleotides they are usually called Gap-mers, if the DNA segment is flanked by the segments of the DNA analogue, Head-mers, if the segment of the DNA analogue is located in the 5' region of the oligo, and Tail-mers, if the segment of the DNA analogue is located in the 3' region of the oligo.

It should be mentioned that other important mechanisms are involved in Antisense that are not dependent on RNase H activation. For such oligonucleotides the DNA analogues, like LNA, can be placed in any combination design (Childs et al. PNAS, 2002, 99(17), 11091-96; Crinelli et al., Nucl. Acid. Res., 2002, 30(11), 2435-43; Elayadi et al., Biochemistry, 2002, in press; Kurreck et al., Nucl. Acid. Res., 2002, 30(9), in press; Alayadi & Corey, Curr. opinion in Inves. Drugs., 2001, 2(4), 558-61; Braasch & Corey, Chem. & Biol., 2000, 55, 1-7).

In contrast to the beta-D-oxy-LNA the alpha-L-oxy-LNA has a DNA-like locked conformation and it has been demonstrated that alpha-L-oxy-LNA can activate RNase H (Sørensen et al., J. Amer. Chem. Soc., 2002, 124(10), 2164-76). However, the cleavage rate of RNase H is much lower compared to DNA in the disclosed designs and thus, the oligonucleotides in the disclosed designs have not been shown to be efficient Antisense reagents.

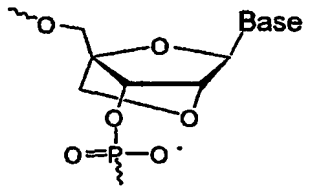
Summary of the Invention

The present inventors have found a novel class of pharmaceuticals which can be used in antisense therapy. Also, the inventors disclose novel oligonucleotides with improved antisense properties. The novel oligonucleotides are composed of at least one Locked Nucleic Acid (LNA) selected from beta-D-thio/amino-LNA or alpha-L-oxy/thio/amino-LNA.. The oligonucleotides comprising LNA may also include DNA and/or RNA nucleotides.

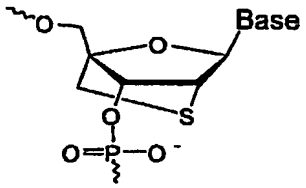
The present inventors have demonstrated that α-L-oxy-LNA surprisingly provides the possibility for the design of improved Antisense oligonucleotides that are efficient substrates for RNase H. These novel designs are not previously described and the guidelines developed broaden the design possibilities of potent Antisense oligonucleotides. Also comprised in this invention is the disclosure of Antisense oligonucleotides having other improved properties than the capability of being RNase H substrates. The oligonucleotides comprise any combination of LNA-relatives with DNA/RNA, and their analogues, as well as oxy-LNA. The design of more potent Antisense reagents is a combination of several features. Among the features of these novel oligonucleotide designs are increased enzymatic stability, increased cellular uptake, and efficient ability to recrute RNase H. Also important is the relation between the length and the potency of the oligonucleotides (e.g. a 15-mer having the same potency as a 21-mer is regarded to be much more optimal). The potency of the novel oligonucleotides comprised in this invention is tested in cellular in vitro assays.

It is furthermore anticipated that the novel designs also improved the in vivo properties such as better pharmacokinetic/pharmacological properties and toxicity profiles.

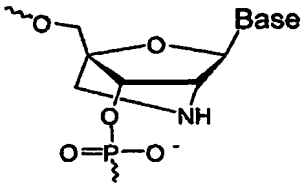
Beta-D-oxy-LNA and the analogues thio-and amino LNA:



Oxy-LNA

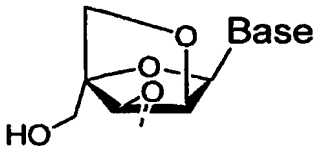


Thio-LNA

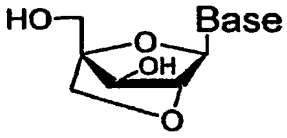


Amino-LNA

LNA diastereoisomers:

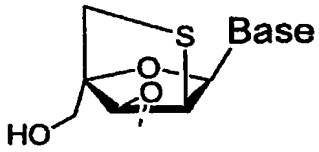


α-L-*oxy*-LNA

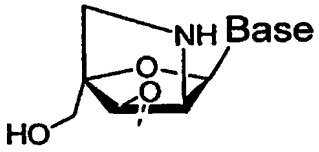


β-D-*xylo*-LNA

α-D-*oxy*-LNA



α-L-*thio*-LNA



α-L-*amino*-LNA

Sugar conformations in DNA:

C 2'-endo (S-type) C 3'-endo (N-type)

Disclosure of the Invention

Thus, the present invention in it broadest scope relates to a pharmaceutical composition comprising a therapeutically active antisense oligonucleotide construct which (i) comprises at least one locked nucleic acid unit selected from the group consisting of amino-LNA and thio-LNA and derivatives thereof; or (ii) comprises at least two consecutively located locked nucleotide units of which at least one is selected from the group consisting of alpha-

L-oxy-LNA and derivatives thereof. The antisense construct can be in the form of a salt or in the form of prodrug or salts of such prodrug. The invention thus relates to pharmaceutical compositions in which an active ingredient is a pharmaceutically acceptable salt, prodrug (such as an ester) or salts of such prodrug of the above oligonucleotide construct. Both amino- and thio-LNA can be either alpha or beta configuration, and in (i), the oligonucleotide construct encompasses constructs with at least one (such as 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) units selected from the group consisting of: alpha-L-thio-LNA, beta-D-thio-LNA, beta-D-amino-LNA, alpha-L-amino-LNA and derivatives thereof; optionally in combination with a least one (such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) further independently selected locked or non-locked nucleotide units. Examples on these further units are oxy-LNA (such as alpha-L or beta-D), thio/amino LNA (such as alpha-L or beta-D), a nucleotide unit which has a 2'-deoxy-erythro-pentofuranosyl sugar moiety (such as a DNA nucleotide), a nucleotide unit which has a ribo-pentofuranosyl sugar moiety (such as a RNA nucleotide); and derivatives thereof. In (ii), the oligonucleotide construct encompasses constructs with at least two (such as 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) consecutively located nucleotide units, of which at least one (such as 1, 2, 3, 4, 5, 6, 7 or more) is alpha-L-oxy LNA units or derivatives thereof. In addition to the alpha-L-oxy LNA units or derivatives thereof, the sequence of consecutively located locked nucleotide units optionally comprises other locked nucleotide units (such as the units defined herein). Besides the essential two consecutively located locked nucleotide units, the construct in (ii) optionally comprises one or more (such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) independently selected locked or non-locked nucleotide units (such as the units defined herein).

In an interesting embodiment, the invention relates to a pharmaceutical composition in which the antisense oligonucleotide construct comprises two adjacently located nucleotide sequences A and B, where

A represents a sequence of nucleotide units comprising (i) at least one locked nucleotide unit selected from the group consisting of thio-LNA, amino-LNA (both in either alpha-L or beta-D configuration) and derivatives thereof, or (ii) at least two consecutively located locked nucleotide units of which at least one is selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof; and

B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl sugar moiety. Sequence A can additionally comprise at least one further locked nucleotide unit (such as 2, 3, 4 or 5 units), preferably selected independently from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D configuration), alpha-L-oxy-LNA and derivatives thereof.

In an other interesting embodiment, the invention relates to a pharmaceutical composition comprising an oligonucleotide construct which contains three adjacently located nucleotide sequences, A, B and C, in the following order (5' to 3'):
A-B-C or C-B-A,
in which
A represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha-L or beta-D configuration) and derivatives thereof;
B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety; and
C represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha-L or beta-D configuration) and derivatives thereof.

The invention also relates to an oligonucleotide construct which comprises at least one nucleotide sequence comprising one or more nucleotide units selected from the group consisting of amino-LNA, thio-LNA (in all configurations) and derivatives thereof;
with the proviso that the following oligonucleotide constructs are excluded:
(I) 5'-d(GTGAVATGC), 5'-d(GVGAVAVGC), 5'-d(GTGAXATGC), 5'-d(GXGAXAXGC), 5'-d(GXGVXVXGC), in which sequences V represents a beta-D-amino-LNA thymine unit, and X represents a beta-D-methylamino-LNA thymine unit; and
(ii) 5'-d(GTGAYATGC), 5'-d(GYGAYAYGC) and 5'-d(GYGYYYYGC) in which sequences Y represents a beta-D-thio-LNA uracil unit.

The excluded oligonucleotides are previously disclosed by Singh et al and Kumar et al. (Kumar et al. *Bioorg, & Med. Chem. Lett.*,1998, 8, 2219-2222; Singh et al. *J. Org. Chem.*, 1998, 63, 10035-39). It has collectively for the excluded LNA- relatives been shown that they can be incorporated into oligonucleotides. However, no biological properties have not been demonstrated or suggested.

A presently preferred group of oligonucleotide constructs of the invention comprises two adjacently located nucleotide sequences, A and B, where A represents a sequence of nucleotide units comprising at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D) configuration, and derivatives thereof; and B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety; especially constructs in which B represents a sequence of nucleotide units, said sequence contains a subsequence of at least three nucleotide units having 2'-deoxy-erythro-pentofuranosyl sugar moieties, such as 4, 5, 6, 7, 8, 9 or 10 nucleotide units, said subsequence optionally being spiked with an other nucleotide, preferably an alpha-L-oxy-LNA unit selected from the group consisting of alpha-L-amino-LNA, alpha-L-thio-LNA, alpha-L-oxy-LNA and derivatives thereof.

Also interesting is a construct according which comprises three adjacently located nucleotide sequences in the following order (5' to 3'):A-B-C,
in which the nucleotide sequences A and B are as defined as above, and C represents a sequence of nucleotide units, which comprises at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D configuration) and derivatives thereof.

In the above constructs, it is preferred that A has a length of 2-10 (preferably 2-8, such as 3, 4, 5, 6, 7) nucleotide units; B has a length of 1-10 (preferably 5-8, such as 6 or 7) nucleotide units; and C (if present) has a length of 2-10 (preferably 2-8, such as 3, 4, 5, 6, or 7) nucleotide units; so that the overall length of the construct is 6-30 (preferably 10-20, more preferably 12-18, such as 13, 14, 15, 16 or 17) nucleotide units.

A preferred embodiment of the above construct according to the invention is a construct in which A represents a sequence of nucleotide units comprising at least two consecutively located locked nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), at least one of said locked nucleotide units being selected from the group consisting of amino-LNA, thio-LNA and derivatives thereof; C represents a sequence of nucleotide units comprising at least two consecutively located locked nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), at least one of said locked nucleotide units being selected from the group consisting of amino-LNA, thio-LNA (in all configurations) and derivatives thereof, and/or B represents a sequence of least 2 nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), which sequence in addition to the nucleotide unit(s) having 2'-deoxy-erythro-pentofuranosyl sugar moiety(ies) and/or ribo-pentofuranosyl moiety(ies), comprises nucleotides units

which are selected independently from the group consisting of: locked nucleotide units (such as alpha-L-oxy-, -thio-, or -amino- nucleotide units) and derivatives thereof.

An other embodiment of the invention relates to an oligonucleotide construct which contains three adjacently located nucleotide sequences, A, B and C, in the following order (5' to 3'): A-B-C or C-B-A, in which
A represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha or beta configuration) and derivatives thereof;
B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety; and
C represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha or beta configuration) and derivatives thereof. It is preferred that A has a length of 2-10 (preferably 2, 3, 4, 5, 6, 7, or 8) nucleotide units; B has a length of 1-10 (preferably 5, 6, 7, or 8) nucleotide units;
C has a length of 2-10 (preferably 2, 3, 4, 5, 6, 7, or 8) nucleotide units; so that the overall length of the construct is 8-30 (preferably 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20) nucleotide units.

An other interesting embodiment is a construct in which A represents a sequence of nucleotide units comprising at least three consecutively located locked nucleotide units, at least one of said locked nucleotide units being selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof; C represents a sequence of nucleotide units comprising at least three consecutively located locked nucleotide units, at least one of said locked nucleotide units being selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof; and/or B represents a sequence of least 2 nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), which sequence in addition to the nucleotide unit(s) having 2'-deoxy-erythro-pentofuranosyl sugar moiety(ies) and/or ribo-pentofuranosyl moiety(ies), comprises nucleotide units which are selected independently from the group consisting of:

locked nucleotide units (such as alpha-L-oxy-, -thio-, or -amino- nucleotide units) and derivatives thereof. Especially preferred is a construct in which A and C comprises at least one alpha-L-oxy-LNA or alpha-L-thio-LNA unit located adjacent to B.

In a further embodiment, the invention relates to an oligonucleotide which has the formula (in 5' to 3' order): A-B-C-D, in which A represents a sequence of locked nucleotide units; B represents a sequence of non-locked nucleotide units, preferably at least one unit has a 2'-deoxy pentofuranose sugar moiety, in which sequence 1 or 2 nucleotide units optionally are substituted with locked nucleotide units, preferably alpha-L-oxy-LNA; C represents a sequence of locked nucleotide units; and D represents a non-locked nucleotide unit or a sequence of non-locked nucleotide units. It is preferred that A has a length of 2-6 (preferably 3, 4 or 5) nucleotide units; B has a length of 4-12 (preferably 6, 7, 8, 9, 10 or 11) nucleotide units; C has a length of 1-5 (preferably 2, 3, or 4) nucleotide units; D has a lenght of 1-3 (preferably 1-2) nucleotide units; and that the overall length of the construct is 8-26 (preferably 12-21) nucleotide units. In presently preferred construct, A has a length of 4 nucleotide units; B has a length of 7-9, preferably 8, nucleotide units; C has a length of 3 nucleotide units; D has a length of 1 nucleotide unit; and the overall length of the construct is 15-17 (preferably 16) nucleotide units. It is further preferred that the locked nucleotide units in A and C are beta-D-oxy-LNA units or derivatives thereof.

The oligonucleotide constructs according to the invention can contain naturally occurring phosphordiester internucleoside linkages, as well as other internucleoside linkages as defined in this specification. Examples on internucleoside linkages are linkages selected from the group consisting of $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-NR^H-P(O)_2-O-$, $-O-P(O,NR^H)-O-$, $-O-PO(R'')-O-$, $-O-PO(CH_3)-O-$, and $-O-PO(NHR^N)-O-$, where R^H is selected form hydrogen and C_{1-4}-alkyl, and R'' is selected from C_{1-6}-alkyl and phenyl.

In a further embodiment, the invention relates to an oligonucleotide construct which comprises at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D configuration), alpha-L-oxy-LNA, and derivatives thereof; wherein at least one of the linkages between the nucleotide units is different from the natural occurring phosphordiester ($-O-P(O)_2-O-$) linker. Constructs in which the internucleoside linkage (between 3' carbon and 5' carbon on adjacent (3', 5' dideoxy) nucleosides) selected from the group consisting of: $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-NR^H-P(O)_2-O-$, $-O-P(O,NR^H)-O-$, $-O-PO(R'')-O-$, $-O-PO(CH_3)-O-$, and $-O-PO(NHR^N)-O-$, where R^H is selected form hydrogen and C_{1-4}-alkyl, and R'' is selected from C_{1-6}-alkyl and phenyl, is presently preferred, and the phoshorothioate internucleoside linkage is presently most preferred.

An embodiment of the oligonucleotide constructs according to the invention relates to such constructs that are able to mediate enzymatic inactivation (at least partly) of the target nucleic acid (eg. a RNA molecule) for the construct. Constructs that mediate RNase H cutting of the target are within the scope of the present invention. Thus, the present invention relates to constructs that are able to recruit RNase, especially constructs in which sequence B represents a sequence of nucleotide units that makes the construct able to recruit RNase H when hybridised to a target nucleic acid (such as RNA, mRNA).

It should be understood that the invention also relates to a pharmaceutical composition which comprises a least one antisense oligonucleotide construct of the invention as an active ingredient. It should be understood that the pharmaceutical composition according to the invention optionally comprises a pharmaceutical carrier, and that the pharmaceutical composition optionally comprises further antisense compounds, chemotherapeutic compounds, antiinflammatory compounds and/or antiviral compounds.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be (a) oral (b) pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, (c) topical including epidermal, transdermal, ophthalmic and to mucous membranes including vaginal and rectal delivery; or (d) parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Compositions and formulations for oral administration include but is not restricted to powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self- emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels and suppositories. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

The antisense nucleotide constructs of the invention encompass, in their brodest scope, any pharmaceutically acceptable salts, esters, or salts of such esters. Furthermore encompasses the invention any other compound, which, upon administration to an animal or a human, is capable of directly or indirectly providing the biologically active metabolite or residue thereof. The invention therefore also encompasses prodrugs of the compounds of the invention and pharmaceutically acceptable salts of such prodrugs, and other bioequivalents. The term prodrug indicates a therapeutic agent that is prepared in an inactive form and that is converted to an active form, a drug, within the body or cells thereof. The pharmaceutically acceptable salts include but are not limited to salts formed with cations; acid addition salts formed with inorganic acids salts formed with organic acids such as, and salts formed from elemental anions.

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals or humans. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of

non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Pharmaceutical compositions of the invention include a pharmaceutical carrier that may contain a variety of components that provide a variety of functions, including regulation of drug concentration, regulation of solubility, chemical stabilization, regulation of viscosity, absorption enhancement, regulation of pH, and the like. The pharmaceutical carrier may comprise a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solutions of dextrose, and the like. For water soluble formulations, the pharmaceutical composition preferably includes a buffer such as a phosphate buffer, or other organic acid salt. For formulations containing weakly soluble antisense compounds, micro-emulsions may be employed. Other components may include antioxidants, such as ascorbic acid, hydrophilic polymers, such as, monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, dextrins, chelating agents, and like components well known to those in the pharmaceutical sciences. The oligonucleotides may be encapsulated in liposomes for therapeutic delivery.

In a certain embodiment, the present invention provides pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g. , mithramycin and oligonucleotide), sequentially (e.g., mithramycin and oligonucleotide for a period of time followed by another agent and oligonucleotide), or in combination with one or more other such chemotherapeutic agents or in combination with radiotherapy.

Anti-inflammatory drugs, including but not limited to nonsteroidal anti- inflammatory drugs and corticosteroids, and antiviral drugs, may also be combined in compositions of the invention. Two or more combined compounds may be used together or sequentially.

In another embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Two or more combined compounds may be used together or sequentially.

Dosing is dependent on severity and responsiveness of the disease state to be treated, and the course of treatment lasting from several days to several months, or until a cure is

effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient.

Optimum dosages may vary depending on the relative potency of individual oligonucleotides. Generally it can be estimated based on EC50s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 25 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 10 years. The repetition rates for dosing can be estimated based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state.

The LNA containing antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal or a human, suspected of having a disease or disorder, which can be treated by modulating the expression of a gene by administering antisense compounds in accordance with this invention. Further provided are methods of treating an animal and humans, suspected of having or being prone to a disease or condition, associated with expression of a target gene by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention. Examples of such a diseases are for example different types of cancer, infectious and inflammatory diseases.

Definitions

The term "nucleotide sequence" or "sequence" comprises a plurality (ie. more than one) nucleosides (or derivatives thereof), in which sequence each two adjacent nucleosides (or derivatives thereof) are linked by an internucleoside linker. When the length of a sequence are defined by a range (such as from 2-10 nucleotide units), the range are understood to comprise all integers in that range, i.e. "a sequence of 2-10 nucleotide units" comprises sequences having 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide units.

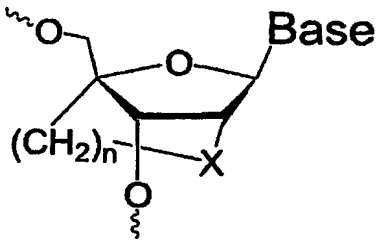
In the present context, the term "oligonucleotide" (or oligo, oligomer) means a successive chain of nucleoside units (i.e. glycosides of heterocyclic bases) connected via internucleoside linkages.

By the term "unit" is understood a monomer.

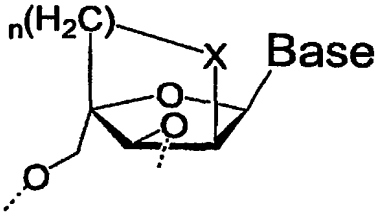
The term "at least one" comprises the integers larger than or equal to 1, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and so forth.

The term "locked nucleotide" comprises nucleotides in which the 2' deoxy ribose sugar moiety is modified by introduction of a structure containing a heteroatom bridging from the 2' to the 4' carbon atoms. The term includes nucleotides having the following substructures (the oxygen at the 3' and 5' ends illustrates examples of the starting point of the internucleoside linkages):

beta-D-LNA derivatives:



alpha-L-LNA derivatives



In both structures, X represents O, S or N-R (R= H; C1-C6 alkyl such as methyl, ethyl, propyl, I-propyl, butyl, i-butyl, t-butyl and pentyl); and
n is an integer 1, 2 or 3, so that the group -(CH2)n- comprises methylen, ethylen or propylen groups. In these alkylene groups (and the -N(C1-C6 alkyl)- group), one or more H atoms can be replaced with substituents, such as one or more substituents selected from the group consisting of halogen atoms (Cl, F, Br, I), Nitro, C1-6 alkyl or C1-6 alkoxy, both optionally halogenated.

In the present context, the term "C_{1-6}-alkyl" means a linear, cyclic or branched hydrocarbon group having 1 to 6 carbon atoms, such as methyl, ethyl, propyl, *iso*-propyl, butyl, *tert*-butyl, *iso*-butyl, pentyl, cyclopentyl, hexyl, cyclohexyl, in particular methyl, ethyl, propyl, *iso*-propyl, *tert*-butyl, *iso*-butyl and cyclohexyl. "C_{1-6}-alkoxy" means -O-(C1-6-alkyl).

The term "non-locked nucleotide" comprises nucleotides that do not contain a bridging structure in the ribose sugar moiety. Thus, the term comprises DNA and RNA nucleotide monomers (phosphorylated adenosine, guanosine, uridine, cytidine, deoxyadenosine, deoxyguanosine, deoxythymidine, deoxycytidine) and derivatives thereof as well as other

nucleotides having a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety.

The term "thio-LNA" comprises a locked nucleotide in which X in the above formulas represents S, and n is 1. Thio-LNA can be in both beta-D and alpha-L-configuration.

The term "amino-LNA" comprises a locked nucleotide in which X in the above formulas represents -NR-, and n is 1. Amino-LNA can be in both beta-D and alpha-L-configuration.

The term "oxy-LNA" comprises a locked nucleotide in which X in the above formulas represents O and n is 1. Oxy-LNA can be in both beta-D and alpha-L-configuration.

By the term "alpha-L-LNA" as used herein is normally understood alpha-L-oxy-LNA (n=1 in the bridging group), and by the term "LNA" as used herein is understood beta-D-oxy-LNA monomer wherein n in the bridging group is 1.

However, derivatives of the above locked LNA's comprise nucleotides in which n is an other integer than 1.

By the term "derivatives thereof" in connection with nucleotides (e.g. LNA and derivatives thereof) is understood that the nucleotide, in addition to the bridging of the furan ring, can be further derivatized. For example, the base of the nucleotide, in addition to adenine, guanine, cytosine, uracil and thymine, can be a derivative thereof, or the base can be substituted with other bases. Such bases includes heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are xanthine, diaminopurine, 8-oxo-N^6-methyladenine, 7-deazaxanthine, 7-deazaguanine, N^4,N^4-ethanocytosin, N^6,N^6-ethano-2,6-diaminopurine, 5-methylcytosine, 5-(C^3-C^6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanin, inosine, N^6-alylpurines, N^6-acylpurines, N^6- benzylpurine, N^6-halopurine, N^6-vinylpurine, N^6-acetylenic purine, N^6-acyl purine, N^6-hydroxyalkyl purine, N^6-thioalkyl purine, N^2-alkylpurines, N^4-alkylpyrimidines, N^4- acylpyrimidines, N^4-benzylpurine, N^4-halopyrimidines, N^4-vinylpyrimidines, N^4 - acetylenic pyrimidines, N^4-acyl pyrimidines, N^4-hydroxyalkyl pyrimidines, N^6-thioalkyl pyrimidines, thymine, cytosine, 6-azapyrimidine, including 6-azacytosine, 2- and/or 4- mercaptopyrimidine, uracil, C^5-alkylpyrimidines, C^5-benzylpyrimidines, C^5-halopyrimidines, C^5-vinylpyrimidine, C^5-acetylenic pyrimidine, C^5-acyl pyrimidine, C^5-hydroxyalkyl purine, C^5- amidopyrimidine, C^5-cyanopyrimidine, C^5-nitropyrimidine, C^5-aminopyrimdine, N^2- alkylpurines, N^2-alkyl-6-thiopurines, 5-azacytidinyl, 5- azauracilyl, trazolopyridinyl, imidazolopyridinyl, pyrrolopyrimidinyl, and pyrazolopyrimidinyl. Functional oxygen and nitrogen groups on the base can be protected

as necessary or desired. Suitable protecting groups are well known to those skilled in the art, and included trimethylsilyl, dimethylhexylsilyl, *t*-butyldimenthylsilyl, and *t*-butyldiphenylsilyl, trityl, alkyl groups, acyl groups such as acetyl and propionyl, methanesulfonyl, and p-toluenesulfonyl. Preferred bases include cytosine, methyl cytosine, uracil, thymine, adenine and guanine. In addition to the derivatisation of the base, both locked and non-locked nucleotides can be derivatised on the ribose moiety. For example, a 2' substituent can be introduced, such as a substituent selected from the group consisting of halogen (such as fluor), C1-C9 alkoxy (such as methoxy, ethoxy, n-propoxy or i-propoxy), C1-C9 aminoalkoxy (such as aminomethoxy and aminoethoxy), allyloxy, imidazolealkoxy, and polyethyleneglycol, or a 5' substituent (such as a substituent as defined above for the 2' position) can be introduced.

By the terms "internucleoside linkage" and "linkage between the nucleotide units" (which is used interchangeably) are to be understood the divalent linker group that forms the covalent linking of two adjacent nucleosides, between the 3' carbon atom on the first nucleoside and the 5' carbon atom on the second nucleoside (said nucleosides being 3',5' dideoxy). The oligonucleotides of the present invention comprises sequences in which both locked and non-locked nucleotides independently can be derivatised on the internucleoside linkage which is a linkage consisting of preferably 2 to 4 groups/atoms selected from $-CH_2-$, $-O-$, $-S-$, $-NR^H-$, $>C=O$, $>C=NR^H$, $>C=S$, $-Si(R'')_2-$, $-SO-$, $-S(O)_2-$, $-P(O)_2-$, $-PO(BH_3)-$, $-P(O,S)-$, $-P(S)_2-$, $-PO(R'')-$, $-PO(OCH_3)-$, and $-PO(NHR^H)-$, where R^H is selected form hydrogen and C_{1-6}-alkyl, and R'' is selected from C_{1-6}-alkyl and phenyl. Illustrative examples of such internucleoside linkages are $-CH_2-CH_2-CH_2-$, $-CH_2-CO-CH_2-$, $-CH_2-CHOH-CH_2-$, $-O-CH_2-O-$, $-O-CH_2-CH_2-$, $-O-CH_2-CH(R5)-$, $-CH_2-CH_2-O-$, $-NR^H-CH_2-CH_2-$, $-CH_2-CH_2-NR^H-$, $-CH_2-NR^H-CH_2-$, $-O-CH_2-CH_2-NR^H-$, $-NR^H-CO-O-$, $-NR^H-CO-NR^H-$, $-NR^H-CS-NR^H-$, $-NR^H-C(=NR^H)-NR^H-$, $-NR^H-CO-CH_2-NR^H-$, $-O-CO-O-$, $-O-CO-CH_2-O-$, $-O-CH_2-CO-O-$, $-CH_2-CO-NR^H-$, $-O-CO-NR^H-$, $-NR^H-CO-CH_2-$, $-O-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-$, $-CH=N-O-$, $-CH_2-NR^H-O-$, $-CH_2-O-N(R5)-$, $-CH_2-O-NR^H-$, $-CO-NR^H-CH_2-$, $-CH_2-NR^H-O-$, $-CH_2-NR^H-CO-$, $-O-NR^H-CH_2-$, $-O-NR^H-$, $-O-CH_2-S-$, $-S-CH_2-O-$, $-CH_2-CH_2-S-$, $-O-CH_2-CH_2-S-$, $-S-CH_2-CH(R5)-$, $-S-CH_2-CH_2-$, $-S-CH_2-CH_2-O-$, $-S-CH_2-CH_2-S-$, $-CH_2-S-CH_2-$, $-CH_2-SO-CH_2-$, $-CH_2-SO_2-CH_2-$, $-O-SO-O-$, $-O-S(O)_2-O-$, $-O-S(O)_2-CH_2-$, $-O-S(O)_2-NR^H-$, $-NR^H-S(O)_2-CH_2-$, $-O-S(O)_2-CH_2-$, $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-S-P(O)_2-O-$, $-S-P(O,S)-O-$, $-S-P(S)_2-O-$, $-O-P(O)_2-S-$, $-O-P(O,S)-S-$, $-O-P(S)_2-S-$, $-S-P(O)_2-S-$, $-S-P(O,S)-S-$, $-S-P(S)_2-S-$, $-O-PO(R'')-O-$, $-O-PO(OCH_3)-O-$, $-O-PO(OCH_2CH_3)-O-$, $-O-PO(OCH_2CH_2S-R)-O-$, $-O-PO(BH_3)-O-$, $-O-PO(NHR^N)-O-$, $-O-P(O)_2-NR^H-$, $-NR^H-P(O)_2-O-$, $-O-P(O,NR^H)-O-$, $-CH_2-P(O)_2-O-$, $-O-P(O)_2-CH_2-$, and $-O-Si(R'')_2-O-$; where R5 is selected from hydrogen and C_{1-6}-alkyl, R^H is selected form hydrogen and C_{1-6}-alkyl, and R'' is selected from C_{1-6}-alkyl and phenyl.

$-CH_2-CO-NR^H-$, $-CH_2-NR^H-O-$, $-S-CH_2-O-$, $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-NR^H-P(O)_2-O-$, $-O-P(O,NR^H)-O-$, $-O-PO(R'')-O-$, $-O-PO(CH_3)-O-$, and $-O-PO(NHR^N)-O-$, where R^H is selected from hydrogen and C_{1-6}-alkyl, and R'' is selected from C_{1-6}-alkyl and phenyl, are especially preferred.

The nucleotides units may also contain a 3'-Terminal group or a 5'-terminal group, preferably –OH.

By the term "able to recruit RNase H" is understood that the an oligonucleotide construct, in order to elicit RNase H enzyme cleavage of a target nucleic acid (such as target mRNA), must include a segment or subsequence that is of DNA type. This means that at least some nucleotide units of the oligonucleotide construct (or a subsequence thereof) must have 2'-deoxy-erythro-pentofuranosyl sugar moieties. A subsequence having more than three consecutive, linked 2'-deoxy-erythro-pentofuranosyl containing nucleotide units likely is necessary in order to elicit RNase H activity upon hybridisation of an oligonucleotide construct of the invention with a target nucleic acid, such as a RNA. Preferably, a sequence which is able to recruit RNase H contains more than three consecutively located nucleotides having 2'-deoxy-erythro-pentofuranosyl sugar moieties, such as 4,5, 6, 7, 8 or more units. However, such a subsequence of consecutively located nucleotides having 2'-deoxy-erythro-pentofuranosyl sugar moieties can by spiked (ie. one or more (such as 1, 2, 3, 4, or more) nucleotides being replaced) with other nucleotides, preferably alpha-L-oxy, thio- or amino-LNA units or derivatives thereof.

The term "pharmaceutically acceptable salt" is well known to the person skilled in the art.

Examples of such pharmaceutically acceptable salts are the iodide, acetate, phenylacetate, trifluoroacetate, acrylate, ascorbate, benzoate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, bromide, isobutyrate, phenylbutyrate, g-hydroxybutyrate, b-hydroxybutyrate, butyne-1,4-dioate, hexyne-1,4-dioate, hexyne-1,6-dioate, caproate, caprylate, chloride, cinnamate, citrate, decanoate, formate, fumarate, glycollate, heptanoate, hippurate, lactate, malate, maleate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, isonicotinate, nitrate, oxalate, phthalate, terephthalate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, propiolate, propionate, phenylpropionate, salicylate, sebacate, succinate, suberate, sulfate, bisulfate, pyrosulfate, sulfite, bisulfite, sulfonate, benzenesulfonate, p-bromophenylsulfonate, chlorobenzenesulfonate, propanesulfonate, ethanesulfonate, 2-hydroxyethanesulfonate, methanesulfonate, naphthalene-I -sulfonate, naphthalene-2-sulfonate, p-toluenesulfonate, xylenesulfonate, tartarate, and the like.

Legends to figures

Figure 1: Stability of oligonucleotides containing beta-D-amino-LNA against SVPD. (Capital letters are LNA, T^N stands for beta-D-amino-LNA and small letters are DNA. The oligonucleotide is synthesized on deoxynucleoside-support, t.)

Figure 2: Down-regulation of Luciferase expression of oligonucleotides gapmers containing beta-D-amino-LNA or beta-D-thio-LNA and the corresponding beta-D-oxy-LNA gapmer control at 50 nM oligonucleotide concentration.

Figure 3: Electrophoresis analysis of ^{32}P-labelled target RNA degradation products mediated by RnaseH and an oligonucleotide containing beta-D-amino-LNA. Aliquots taken at 0, 10, 20 and 30 min for each design. In the drawings, the line is DNA, the rectangle beta-D-amino- or -thio-LNA.

Figure 4: Stability of oligonucleotides containing beta-D-thio-LNA against SVPD. (Capital letters are LNA, T^S stands for beta-D-thio-LNA and small letters are DNA. The oligonucleotide is synthesized on deoxynucleoside-support, t.)

Figure 5: FACS analysis of oligonucleotides containing beta-D-thio-LNA and the corresponding controls.

Figure 6: Stability of oligonucleotides containing alpha-L-oxy-LNA against SVPD. (Capital letters are LNA, T^a stands for alpha-L-oxy-LNA and small letters are DNA. The oligonucleotide is synthesized on deoxynucleoside-support, t.)

Figure 7: Stability of different oligonucleotides (t_{16}, t_{s12}, T_{16}, $T^a{}_{15}T$) against S1-endonuclease. (Capital letters are LNA, T^a stands for alpha-L-oxy-LNA and small letters are DNA. The oligonucleotide is synthesized on oxy-LNA-support, T.)

Figure 8: Gapmers including alpha-L-oxy-LNA (shadowed in gray).

Figure 9: Down-regulation of Luciferase expression of oligonucleotides containing alpha-L-oxy-LNA at 50nM oligonucleotide concentration.

Figure 10: Different mixmers containing alpha-L-oxy-LNA. The numbers stand for the alternate contiguous stretch of DNA or LNA. In the drawing, the line is DNA, the rectangle beta-D-oxy-LNA, the gray shadow corresponds to alpha-L-oxy-LNA residues.

Figure 11: Other mixmers containing alpha-L-oxy-LNA. The numbers stand for the alternate contiguous stretch of DNA or alpha-L-oxy-LNA. In the drawing, the line is DNA, the gray shadow corresponds to alpha-L-oxy-LNA residues.

Figure 12: Electrophoresis analysis of ^{32}P-labelled target RNA degradation products mediated by RnaseH and an oligonucleotide containing alpha-L-oxy-LNA. Aliquots taken at 0, 10, 20 and 30 min for each design. In the drawings, the line is DNA, the rectangle beta-D-oxy-LNA, the gray shadow corresponds to alpha-L-oxy-LNA residues.

Figure 13: FACS analysis of oligonucleotides containing alpha-L-oxy-LNA, and the corresponding controls.

Figure 14: Special constructs with beta-D- oxy-LNA. The numbers stand for the alternate contiguous stretch of DNA and beta-D-oxy-LNA. In the drawing, the line is DNA, the rectangle is beta-D-oxy-LNA.

Figure 15: Down-regulation of Luciferase expression of special constructs containing beta-D-oxy-LNA (designs 3-9-3-1) at 2nM oligonucleotide concentration.

Experimental

Oligonucleotide synthesis

Oligonucleotides were synthesized using the phosphoramidite approach on an Expedite 8900/MOSS synthesizer (Multiple Oligonucleotide Synthesis System) at 1 µM scale. At the end of the synthesis (DMT-on) the oligonucleotides were cleaved from the solid support using aqueous ammonia for 1 h at room temperature, and further deprotected for 4 h at 65°C. The crudes were purified by reverse phase HPLC. After the removal of the DMT-group, the oligonucleotides were characterized by AE-HPLC or RP-HPLC, and the structure further confirmed by ESI.

3'-Exonuclease stability study

Snake venom phosphodiesterase (SVPD, Amersham Pharmacia) assays were performed using 26 µg/mL oligonucleotide, 0.3 µg/mL enzyme at 37°C in a buffer of 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8. The enzyme was shown to maintain its activity under these conditions for at least 2 h. Aliquots of the enzymatic digestion were removed at the indicated times, quenched by heat denaturation for 3 min and stored at -20°C until analysis by RP-HPLC.

S1-Endonuclease stability study

S1 endonuclease (Amersham Pharmacia) assays were performed using 1.5 µmol oligonucleotide and 16 U/mL enzyme at 37°C in a buffer of 30 mM NaOAc, 100 mM NaCl, 1 mM $ZnSO_4$, pH 4.6. The enzyme was shown to maintain its activity under these conditions for at least 2 h. Aliquots of the enzymatic digestion were removed at the indicated times, quenched by freezing-drying, and stored at -20°C until analysis by either RP-HPLC and ES-MS or polyacrylamide electrophoresis.

Luciferase assayThe X1/5 Hela cell line (ECACC Ref. No: 95051229), which is stably transfected with a "tet-off" luciferase system, was used. In the absence of tetracycline the luciferase gene is expressed constitutively. The expression can be measured as light in a luminometer, when the luciferase substrate, luciferin has been added.

The X1/5 Hela cell line was grown in Minimun Essential Medium Eagle (Sigma M2279) supplemented with 1x Non Essential Amino Acid (Sigma M7145), 1x Glutamax I (Invitrogen 35050-038), 10 % FBS calf serum, 25 µg/ml Gentamicin (Sigma G1397), 500 µg/ml G418 (Invitrogen 10131-027) and 300 µg/ml Hygromycin B (invitrogen 10687-010). The X1/5 Hela cells were seeded at a density of 8000 cells per well in a white 96 well plate (Nunc 136101) the day before the transfection. Before the transfection, the cells were washed one time with OptiMEM (Invitrogen) followed by addition of 40 µl of OptiMEM with 2µg/ml of Lipofectamine2000 (Invitrogen). The cells were incubated for 7 minutes before addition of the oligonucleotides. 10 µl of oligonucleotide solutions were added and the cells were incubated for 4 hours at 37°C and 5 % CO_2. After the 4 hours of incubation the cells were washed once in OptiMEM and growth medium was added (100 µl). The luciferase expression was measure the next day.

Luciferase expression was measured with the Steady-Glo luciferase assay system from Promega. 100 µl of the Steady-Glo reagent was added to each well and the plate was shaken for 30s at 700rpm. The plate was read in Luminoskan Ascent instrument from ThermoLabsystems after 8minof incubation to complete total lysis of the cells. The luciferase expression is measured as Relative Light Units per seconds (RLU/s). The data was processed in the Ascent software (v2.6) and graphs were drawn in SigmaPlot2001.

RNaseH assay

25 nM RNA was incubated in the presence of a 10-fold excess of various complementary oligonucleotides in 1 x TMK-glutamate buffer (20 mM Tris acetate, 10 mM magnesium acetate and 200 mM potassium glutamate, pH 7.25) supplied with 1 mM DTT in a reaction volume of 40 µl. The reactions were preincubated for 3 minutes at 65°C followed by 15 minutes at 37°C before addition of RNase H (Promega, Cat.# 4285). 0.2 U of RNase H was added, and samples were withdrawn (6 µl) to formamide dye (3 µl) on ice at the time points 0, 10, 20 and 30 minutes after RNase H addition. 3 µl of the 0, 10, 20 and 30 minutes samples were loaded on a 15 % polyacrylamide gel containing 6M urea and 0.9 x

Tris borate/EDTA buffer. The gel was 0.4 mm thick and ran at 35 watt as the limiting parameter for 2 hours. The gel was dried for 60 minutes at 80°C, followed by ON exposure on Kodak phosphorscreen. The Kodak phosphorscreen was read in a Bio-Rad FX instrument and the result was analysed in Bio-Rad software Quantity One.

Cellular assay

Cell Culture: Cell lines 15PC3 (human prostate cancer) and X1/5 (HeLa cells stably transfected with a Tet-Off luciferase construct) were used, 15PC3 were kindly donated by F. Baas, Neurozintuigen lab, Amsterdam, The Netherlands, X1/5 were purchased from ECACC. 15PC3 were maintained in DMEM + 10% FCS + glutamax + gentamicin and X1/5 were maintained in DMEM + 10% FCS + glutamax + gentamicin + hygromycin + G418 and both cell lines were passaged twice weekly.

Transfection: Cells were seeded at 150000 cells pr. well in 12-well plates the day before transfection.

For transfection with lipid, Lipofectamine 2000 (GIBCO BRL) was mixed with OptiMem and 300 µl of the mixture was added to each well and incubated for 7 min. before addition of 100µl oligo diluted in OptiMem. For each cell line, the optimal Lipofectamine 2000 was determined, for X1/5, the optimal Lipofectamine concentration was 2 µg/ml and for 15PC3 the optimal concentration was 10 µg/ml.

For transfection without lipid, the cells were washed in OptiMem (GIBCO BRL) and 300 µl OptiMem was added to each well. Working stocks of 200 µM were prepared of each oligonucleotide to be tested and added to each well obtaining the desired concentration. For mock controls, oligonucleotide was substituted with water in both protocols.

The cells were incubated with the oligonucleotide for 4 h at 37°C and 5% CO_2 in a humidified atmosphere and subsequently washed in OptiMem before complete growth medium was added. The cells were incubated for an additional 20h.

For FACS analysis, cells were harvested by trypsination and washed twice in Cell Wash (BD) and resuspended in 1x Cell Fix (BD).

FACS analysis: FACS analysis was performed on a FACSCalibur (BD), settings were adjusted on mock controls. Data analysis was performed using the Cell Quest Pro software (BD).

Results

Beta-D-Amino-LNA

Nuclease stability

One of the major difficulties encountered using the naturally occurring phosphodiester oligonucleotides as antisense probes is their rapid degradation by various nucleolytic activities in cells, serum, tissues or culture medium. Since the phosphorus center is the

site of nucleolytic attack, many modifications have been introduced in the internucleoside linkage to prevent enzymatic degradation. To date, the most commonly employed synthetic modification is the backbone phosphorothioate analogue, made by replacing one of the non-bridging oxygen atoms of the internucleoside linkage by sulfur.

We wanted to evaluate the effect of introducing the novel LNA within an oligonucleotide in the presence of nucleases, and to compare it with the well-studied phosphorothioate oligonucleotides. The study was carried out with oligothymidylates by blocking the 3'-end with the novel LNA relatives. The oligonucleotide is synthesized on deoxynucleoside-support (t).

From figure 1, we can appreciate the stability properties, which confer beta-D-amino-LNA. Oligonucleotides containing T-monomer of 2'-beta-D-amino-LNA (T^N) present a remarkable stability against a 3'-exonuclease. Blocking the 3'-end with just two T^N stops the enzyme from degrading the oligonucleotide at least for 2 h. See figure 1.

Luciferase assay: Antisense activity assay

It has been shown that beta-D-oxy-LNA does not elicit RNaseH activity, which is the most common mode of action for an antisense oligonucleotide targeting the down-stream region of the mRNA. However, this disadvantage can be overcome by creating chimeric oligonucleotides composed of beta-D-oxy-LNA and a DNA gap positioned in the middle of the sequence. A gapmer is based on a central stretch of 4-12 DNA (gap) typically flanked by 1 to 6 residues of 2'-*O* modified nucleotides (beta-D-oxy-LNA in our case, flanks).
It was of our interest to evaluate the antisense activity of oligonucleotides, which contain beta-D-amino-LNA in a gapmer design, and compare them with beta-D-oxy-LNA/DNA gapmers.
The oligonucleotides from table 1 were prepared. We decided to carry out the study with gapmers of 16nt in length and a gap of 7nt, which contain 4 residues of beta-D-amino-LNA in one flank and 4 residues of beta-D-oxy-LNA in the other flank, and a thiolated gap. The FAM group was shown not to affect the antisense ability of the oligonucleotides. Therefore, we prepared a FAM-labelled oligonucleotide to be both tested in the Luciferase assay, and in the Cellular uptake.
The oligonucleotide, which targets a motif of the mRNA of the firefly luciferase, contains two mismatches in the flanks. Two C residues of the 5'-end LNA flank were substituted for two Ts for synthetic reasons. At that point in time, only the T residues were available. Therefore and in order to be able to establish a correct comparison, the corresponding beta-D-oxy-LNA control was also included in the assay. No FAM labelling was necessary in this case.

ref	sequence	design	size
U-14	FAM–$T^NT^NT^NT^Ng_st_sc_sa_st_sc_sg_s$TCTTT	Amino-LNA in one flank/ PS gap of 7	16mer
2023-m; 02579	TTTT$g_st_sc_sa_st_sc_sg_s$TCTTT	Control with oxy-LNA	16mer

Table 1 Oligonucleotide containing beta-D-amino-LNA used in the antisense activity assay and the oxy-LNA control (Capital letters for LNA and small letters for DNA, T^N is beta-D-amino-LNA).

From figure 2, we can see that the oligonucleotide with beta-D-amino-LNA presents good antisense activity at 50 nM oligonucleotide concentration. The inclusion of beta-D-amino-LNA in the flanks of an oligonucleotide results in good down-regulation. We can conclude that the antisense activity of an oligonucleotide containing beta-D-amino-LNA is at least as good as the parent all beta-D-oxy-LNA gapmer.

RnaseH assay

RNaseH is a ubiquitous cellular enzyme that specifically degrades the RNA strand of DNA/RNA hybrids, and thereby inactivates the mRNA toward further cellular metabolic processes. The inhibitory potency of some antisense agents seems to correlate with their ability to elicit ribonuclease H (RNaseH) degradation of the RNA target, which is considered a potent mode of action of antisense oligonucleotides. As such, understanding the mechanisms of catalytic function and substrate recognition for the RNaseH is critical in the design of potential antisense molecules.

It was our aim to evaluate the RNaseH activity of gapmers containing beta-D-amino-LNA. From figure 3, we can appreciate a good cleavage activity for an oligonucleotide containing beta-D-amino-LNA.

Beta-D-Thio-LNA

Nuclease stability

As we did for beta-D-amino-LNA, beta-D-thio-LNA was also evaluated against a 3'-exonuclease (SVPD). The oligonucleotide is synthesized on deoxynucleoside-support (t). The study was carried out with oligothymidylates by blocking the 3'-end with beta-D-thio-LNA.

From figure 4, we can see that the incorporation of just one T-monomer of 2'-beta-D-thio-LNA (T^S) has a significant effect in the nucleolytic resistance of the oligonucleotide towards SVPD. After 2h digestion more than 80% of the oligonucleotide remains, while the corresponding beta-D-oxy-LNA oligonucleotide is digested by the exonuclease, see figure 4.

Luciferase assay: Antisense activity assay

We also introduced beta-D-thio-LNA in a gapmer design, and evaluated it in terms of antisense activity.

The oligonucleotides from table 2 were prepared. We decided to carry out the study with gapmers of 16nt in length and a gap of 7nt, which contain 4 residues of beta-D-thio-LNA in one flank and 4 residues of oxy-LNA in the other flank, and a thiolated gap.

The FAM group was shown not to affect the antisense ability of the oligonucleotides. Therefore, we prepared a FAM-labelled oligonucleotide to be both tested in the Luciferase assay, and in the Cellular uptake.

The oligonucleotide, which is directed against a motif of the mRNA of the firefly luciferase, contains two mismatches in the flanks. Two C residues of the 5'-end LNA flank were substituted for two Ts for synthetic reasons. At that point in time, only the T residues were available. Therefore and in order to be able to establish a correct comparison, the corresponding oxy-LNA control was also included in the assay. No FAM labelling was necessary in this case.

ref	sequence	design	size
U-16	$T^{S}T^{S}T^{S}T^{S}g_{s}t_{s}c_{s}a_{s}t_{s}c_{s}g_{s}T^{m}$CTTT-FAM	**Thio-LNA in one flank/ PS gap of 7**	**16mer**
2023-m; 02579	TTTTg$_{s}$t$_{s}$c$_{s}$a$_{s}$t$_{s}$c$_{s}$g$_{s}$TCTTT	**Control with oxy-LNA**	**16mer**

Table 2 Oligonucleotide containing beta-D-thio-LNA used in the antisense activity assay and the corresponding oxy-LNA control (Capital letters for LNA and small letters for DNA, T^{S} is beta-D-thio-LNA).

From figure 2, it can be seen that the oligonucleotide with beta-D-thio-LNA presents good antisense activity at 50 nM oligonucleotide concentration. Therefore, the inclusion of beta-D-thio-LNA in the flanks of an oligonucleotide results in good down-regulation, and is at least as good as the parent all beta-D-oxy-LNA gapmer.

RnaseH assay

We also evaluated gapmer designs that contain beta-D-thio-LNA for their ability to recruit RNaseH activity.

From figure 3, we can see that a beta-D-thio-LNA gapmer recruits RnaseH activity.

Cellular uptake

The efficiency of FAM-labelled oligonucleotide uptake was measured as the mean fluorescence intensity of the transfected cells by FACS analysis.

The transfection without lipid showed distinct differences between the tested oligonucleotides. The uptake as measured from mean fluorescence intensity of transfected cells was dose dependent.

Gapmers (16nt in length and gap of 7nt) containing beta-D-thio-LNA in the flanks were analysed and compared with the corresponding beta-D-oxy-LNA gapmers. Beta-D-thio-LNA (one flank with beta-D-thio-LNA and the other one with oxy-LNA) showed higher uptake than oligonucleotides containing only oxy-LNA. The beta-D-thio-LNA oligonucleotides (both all-PO gapmer and gapmer with PS-gap and PO-flanks) had good uptake efficiency. Specially, the all-PO gapmer containing beta-D-thio-LNA was far superior to other all-PO oligonucleotides tested so far, as it can be appreciated from figure 5.

Alpha-L-oxy LNA

Nuclease stability

The stabilization properties of alpha-L-oxy-LNA were also evaluated. The study was carried out with oligothymidylates by blocking the 3'-end with alpha-L-oxy-LNA. The oligonucleotide is synthesized on deoxynucleoside-support (t). From figure 6, we can see that the introduction of just one alpha-L-T (T^{α}) at the 3'-end of the oligonucleotide represents already a gain of 40% stability (after 2h digestion) with respect to the oxy-version, for which there was actually no gain. The addition of two modifications contributes even more to the stability of the oligonucleotide.

Furthermore, we investigated the effect on stability against S1-endonuclease of alpha-L-oxy-LNA for a 16mer fully modified oligothymidylates. The increased stability of these modified oligonucleotides relative to their deoxynucleotide and phosphorothioate backbone relatives was compared in order to carefully assess the contribution of the alpha-L-oxy-LNA modification.

After 2 h digestion, most of the alpha-L-oxy-LNA oligonucleotide remained (over 80% of the full-length product remained), while neither the oligodeoxynucleotide nor the DNA phosphorothioate analogue could be detected after 30 min digestion (see figure 7). The same kinetic study against S1-endonuclease was carried out with a fully modified oxy-LNA oligonucleotide, which was also very resistant against the S1-endonuclease. Over an 85% of the full-length product remained after 2 h digestion (see figure 7).

In conclusion, beta-D-oxy-LNA, beta-D-amino-LNA, beta-D-thio-LNA and alpha-L-oxy-LNA stabilize oligonucleotides against nucleases. An order of efficiency in stabilization can be established: DNA phosphorothioates << oxy-LNA < α-L-oxy-LNA < beta-D-amino-LNA < beta-D-thio-LNA.

Luciferase assay: antisense activity

Gapmers containing alpha-L-oxy-LNA

We also wanted to see the antisense acticvity in a gapmer oligonucleotides containing alpha-L-oxy-LNA (16nt in length with a thiolated 7nt gap). Two different designs were evaluated.

First, we substituted two oxy-LNA residues for two alpha-L-oxy-LNAs in a gapmer against a motif of the mRNA of the firefly luciferase, and placed the alpha-L-oxy-LNA in the junctions, see figure 8.

Then, we substituted both flanks with alpha-L-oxy-LNA in the same construct, see figure 8.

Previously, different oligonucleotides were tested and compared with the corresponding FAM-labelled molecules, and no significant difference was appreciated between the free and FAM-labelled ones. Therefore, we included oligonucleotides from the Cellular Uptake assay in the Luciferase assay study, assuming that the antisense activity will not be affected by the presence of the FAM group.

From figure 9, the oligonucleotide with alpha-L-oxy-LNA in the junctions shows potent antisense activity. It is actually 5-fold better than the corresponding all oxy-LNA gapmer (gap of 7nt), and slightly better than a gapmer with an optimised 9nt gap with oxy-LNA. The second design (all alpha-L-oxy-LNAs in both flanks) presents at least as good down-regulation levels as the observed for beta-D-oxy-LNA gapmers. We can also conclude that the presence of the alpha-L-oxy-LNA in a gapmer construct shows good-antisense activity level.

alpha-L-oxy-LNA reveals to be a potent tool enabling the construction of different gapmers, which show good antisense activity. The placement of alpha-L-oxy-LNA in the junctions results in a very potent oligonucleotide.

Short-sized gapmers containing alpha-L-oxy-LNA

As a general rule, the length of the construct is usually designed to range from 15-25 nucleotide units, in order to ensure that optimal identification and binding takes place with a unique sequence in the mammalian genome and not with similar genetically redundant elements. Statistical analyses specify 11-15 base paired human sequences as the theoretical lower limits for sufficient recognition of a single genomic region. In practice, however, a longer oligonucleotide is commonly used to compensate for low melting transitions, especially for thiolated oligonucleotides that have lower affinity.

As a significant increase in affinity is achieved by the introduction of oxy-LNA or novel LNA relatives, the design of potent and short antisense oligonucleotides (<15nt) should be enabled.

The alpha-L-oxy-LNA can play an important role in enabling the design of short molecules by maintaining the required high-affinity, but also an optimal gap size. 12 and 14mers against a motif of the mRNA of the firefly luciferase were evaluated.

The results are shown in figure 9. The presence of alpha-L-oxy-LNA in the flanks of a 12 (gap of 7nt) and 14 mer (gap of 8nt) correspond to good levels of down-regulation. From figure 9.

In conclusion, alpha-L-oxy-LNA is a potent tool in enabling the design of short antisense oligonucleotides with significant down-regulation levels.

Mixmers containing alpha-L-oxy-LNA

We also considered other designs containing alpha-L-oxy-LNA against a motif of the mRNA of the firefly luciferase, which we called mixmers. They consist of an alternate composition of DNA, alpha-L-oxy-LNA and beta-D-oxy-LNA. The following figure illustrates the chosen designs. We named the mixmers by the alternate number of units of each alpha-L-oxy-LNA, beta-D-oxy-LNA or DNA composition. See figure 10 and table 3 for the different designs.

ref	sequence	mixmer
2023-q	$TTCCg_sT^a{}_sc_sa_st_sc_sg_sT^a{}_sc_sTTT$	**4-1-1-5-1-1-3 a**
2023-r	$T^aT^aC^aC^ag_sT^a{}_sc_sa_st_sc_sg_sT^a{}_sc_sT^aT^aT$	**4-1-1-5-1-1-3 b**
2023-t	$TTCCg_st_sc_sA^a{}_st_sc_sg_sTCTTT$	**4-3-1-3-5 a**
2023-u	$TTCC^ag_st_sc_sA^a{}_st_sc_sg_sT^aCTTT$	**4-3-1-3-5 b**

Table 3 Mixmers containing alpha-L-oxy-LNA used in this study (Capital letters for LNA and small letters for DNA, T^a is alpha-L-oxy-LNA).

In design **4-1-1-5-1-1-3** (figure 10, table 3), we placed two alpha-L-oxy-LNA residues interrupting the gap, being the flanks beta-D-oxy-LNA. Furthermore, we interrupted the gap with two alpha-L-oxy-LNA residues, and substituted both flanks with alpha-L-oxy-LNA. The presence of alpha-L-oxy-LNA might introduce a flexible transition between the North-locked flanks (oxy-LNA) and the alpha-L-oxy-LNA residue by spiking in deoxynucleotide residues.

It is also interesting to study design **4-3-1-3-5** (figure 10, table 3), where an alpha-L-oxy-LNA residue interrupts the DNA stretch. In addition to the alpha-L-oxy-LNA in the gap, we also substituted two oxy-LNA residues at the edges of the flanks with two alpha-L-oxy-LNA residues.

The presence of just one beta-D-oxy-LNA residue (design **4-3-1-3-5**) interrupting the stretch of DNAs in the gap results in a dramatic loss of down-regulation. Just by using alpha-L-oxy-LNA instead, the design shows significant down-regulation at 50nM oligonucleotide concentration, see figure 9. The placement of alpha-L-oxy-LNA in the

junctions and one alpha-L-oxy-LNA in the middle of the gap also shows down-regulation, see figure 9.

The interruption of the gap with two beta-D-oxy-LNAs (design **4-1-1-5-1-1-3**) relates also with a loss in antisense activity. Again the fully substitution of beta-D-oxy-LNA for alpha-L-oxy-LNA gives significant antisense activity, see figure 9.

alpha-L-oxy-LNA reveals to be a potent tool enabling the construction of different mixmers, which are able to present high levels of antisense activity.

Other designs

Other mixmers containing alpha-L-oxy-LNA were studied, see figure 11.

RnaseH assay

We also evaluated gapmer designs that contain alpha-L-oxy-LNA for their ability to recruit RNaseH activity.

alpha-L-oxy-LNA gapmer and mixmer designs recruit RnaseH activity, see figure 12.

Cellular uptake

The efficiency of FAM-labelled oligonucleotide uptake was measured as the mean fluorescence intensity of the transfected cells by FACS analysis.

The uptake as measured from mean fluorescence intensity of transfected cells was dose dependent.

Gapmers (16nt in length and gap of 7nt) containing α-L-oxy-LNA in the flanks were analysed and compared with the corresponding beta-D-oxy-LNA gapmer.

α-L-oxy-LNA (in both flanks) showed higher uptake than the oligonucleotide containing only beta-D-oxy-LNA. Both all-PO and gapmer with PS-gap had good uptake efficiency; especially the all-PO gapmer was far superior than other all PO oligonucleotides tested so far, see figure 13 for FACS analysis.

Specific beta-D-oxy-LNA constructs

Luciferase assay: Antisense activity assay

Design **3-9-3-1** has a deoxynucleoside residue at the 3'-end, see table 4 and figure 14.

It shows significant levels of down-regulation, in the same range than an optimised (9nt) fully thiolated gapmer. Moreover, only partial thiolation is needed for these mixmers to work as good as the fully thiolated gapmer, see figure 15.

ref	sequence	mixmer
2023-l; 02574	TTCc$_s$g$_s$t$_s$c$_s$a$_s$t$_s$c$_s$g$_s$t$_s$CTTt	3-9-3-1
2023-k; 02575	TTCc$_s$g$_s$t$_s$c$_s$a$_s$t$_s$c$_s$g$_s$t$_s$CTT$_s$t	3-9-3-1
2023-j; 02576	T$_s$T$_s$C$_s$c$_s$g$_s$t$_s$c$_s$a$_s$t$_s$c$_s$g$_s$t$_s$C$_s$T$_s$T$_s$t	3-9-3-1

Table 4 Special beta-D-oxy-LNA constructs (Capital letters for LNA and small letters for DNA).

CLAIMS

1. A pharmaceutical composition comprising a therapeutically active antisense oligonucleotide construct which (i) comprises at least one locked nucleic acid unit selected from the group consisting of amino-LNA and thio-LNA and derivatives thereof; or (ii) comprises at least two consecutively located locked nucleotide units of which at least one is selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof.

2. A pharmaceutical composition according to claim 1, in which the antisense oligonucleotide construct comprises two adjacently located nucleotide sequences A and B, where
A represents a sequence of nucleotide units comprising (i) at least one locked nucleotide unit selected from the group consisting of thio-LNA, amino-LNA (both in either alpha-L or beta-D configuration) and derivatives thereof, or (ii) at least two consecutively located locked nucleotide units of which at least one is selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof; and
B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl sugar moiety.

3. A pharmaceutical composition according to claim 2, in which
sequence A additionally comprises at least one further locked nucleotide unit (such as 2, 3, 4 or 5 units), preferably selected independently from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D configuration), alpha-L-oxy-LNA and derivatives thereof.

4. A pharmaceutical composition according to any of claims 1-2, comprising an oligonucleotide construct which contains three adjacently located nucleotide sequences, A, B and C, in the following order (5' to 3'):
A-B-C or C-B-A,
in which
A represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha-L or beta-D configuration) and derivatives thereof;

B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety; and

C represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha-L or beta-D configuration) and derivatives thereof.

5. A pharmaceutical composition according to any of claims 2-4, in which B represents a sequence of nucleotide units that makes the construct able to recruit RNase H when hybridised to a target nucleic acid.

6. A pharmaceutical composition according to any of claims 1-5, in which the linkages between the nucleotide units in the oligonucleotide construct independently are selected from the group consisting of -O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -NR^H-P(O)$_2$-O-, -O-P(O,NR^H)-O-, -O-PO(R")-O-, -O-PO(CH$_3$)-O-, and -O-PO(NHR^N)-O-, where R^H is selected form hydrogen and C$_{1-6}$-alkyl, and R" is selected from C$_{1-6}$-alkyl and phenyl.

7. A pharmaceutical composition according to any of claims 2-6, in which the linkages between the nucleotides in sequence B in the oligonucleotide construct comprises at least one linkage which is not a -O-P(O)$_2$-O- linkage, such as a phoshorothioate linkage.

8. A pharmaceutical composition according to any of claims 1-7, which further comprises a pharmaceutical carrier.

9. A pharmaceutical compostion according to any of claims 1-8, which further comprises other antisense compounds, chemotherapeutic compounds, antiinflammatory compounds and/or antiviral compounds.

10. An oligonucleotide construct which comprises at least one nucleotide sequence comprising one or more nucleotide units selected from the group consisting of amino-LNA, thio-LNA and derivatives thereof;
with the proviso that the following oligonucleotide constructs are excluded:
(i) 5'-d(GTGAVATGC), 5'-d(GVGAVAVGC), 5'-d(GTGAXATGC), 5'-d(GXGAXAXGC), 5'-d(GXGVXVXGC), in which sequences V represents a beta-D-amino-LNA thymine unit, and X represents a beta-D-methylamino-LNA thymine unit; and

(ii) 5'-d(GTGAYATGC), 5'-d(GYGAYAYGC) and 5'-d(GYGYYYYGC) in which sequences Y represents a beta-D-thio-LNA uracil unit.

11. An oligonucleotide construct according to claim 10, which comprises two adjacently located nucleotide sequences, A and B, where
A represents a sequence of nucleotide units comprising at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D) configuration, and derivatives thereof; and
B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety.

12. An oligonucleotide construct according to any of claims 10-11, which comprises two adjacently located nucleotide sequences, A and B, where
A represents a sequence of nucleotide units comprising at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA and derivatives thereof; and
B represents a sequence of nucleotide units, said sequence contains a subsequence of at least three nucleotide units having 2'-deoxy-erythro-pentofuranosyl sugar moieties, such as 4, 5, 6, 7, 8, 9 or 10 nucleotide units, said subsequence optionally being spiked with an other nucleotide, preferably an alpha-L-oxy-LNA unit selected from the group consisting of alpha-L-amino-LNA, alpha-L-thio-LNA, alpha-L-oxy-LNA and derivatives thereof.

13. A construct according to claim 11-12, comprising the two adjacently sequences in the following order (5' to 3'):

A-B or B-A.

14. A construct according to claim 10-13, which comprises three adjacently located nucleotide sequences in the following order (5' to 3'):

A-B-C,

in which the nucleotide sequences A and B are as defined in any of claims 11-13, and C represents a sequence of nucleotide units, which comprises at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D configuration) and derivatives thereof.

15. A construct according to any of claims 11-14, which is selected from the group consisting of (in 5' to 3' order):

A-B, B-A and A-B-C, where
A, B, and C have the same meaning as defined in claims 11-14, and where
A has a length of 2-10 (preferably 2-8) nucleotide units;
B has a length of 1-10 (preferably 5-8) nucleotide units;
C (if present) has a length of 2-10 (preferably 2-8) nucleotide units; and the overall length of the construct is 6-30 (preferably 10-20, more preferably 12-18) nucleotide units.

16. A construct according to any of claims 11-15, in which A represents a sequence of nucleotide units comprising at least two consecutively located locked nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), at least one of said locked nucleotide units being selected from the group consisting of amino-LNA, thio-LNA and derivatives thereof.

17. A construct according to any of claims 11-16, in which C represents a sequence of nucleotide units comprising at least two consecutively located locked nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), at least one of said locked nucleotide units being selected from the group consisting of amino-LNA, thio-LNA and derivatives thereof.

18. A construct according to any of claims 11-17, in which B represents a sequence of least 2 nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), which sequence in addition to the nucleotide unit(s) having 2'-deoxy-erythro-pentofuranosyl sugar moiety(ies) and/or ribo-pentofuranosyl moiety(ies), comprises nucleotides units which are selected independently from the group consisting of: locked nucleotide units (such as alpha-L-oxy-, -thio-, or -amino- nucleotide units) and derivatives thereof.

19. A construct according to any of claims 10-18, wherein the linkages between the nucleotide units in the oligonucleotide construct independently are selected from the group consisting of $-O-P(O)_2-O-$, $-O-P(O,S)-O-$, $-O-P(S)_2-O-$, $-NR^H-P(O)_2-O-$, $-O-P(O,NR^H)-O-$, $-O-PO(R'')-O-$, $-O-PO(CH_3)-O-$, and $-O-PO(NHR^N)-O-$, where R^H is selected form hydrogen and C_{1-6}-alkyl, and R'' is selected from C_{1-6}-alkyl and phenyl.

20. A construct according to any of claims 11-19, in which the linkages between the nucleotides in sequence B comprises at least one linkage which is not a $-O-P(O)_2-O-$ linkage, such as a phoshorothioate (-O-P(O,S)-O-) linkage.

21. An oligonucleotide construct according to any of claims 11-20, in which
B represents a sequence of nucleotide units that makes the construct able to recruit RNase H when hybridised to a target nucleic acid.

22. An oligonucleotide construct which contains three adjacently located nucleotide sequences, A, B and C, in the following order (5' to 3'):
A-B-C or C-B-A,
in which
A represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha or beta configuration) and derivatives thereof;
B represents one nucleotide unit or a sequence of nucleotide units, with the proviso that at least one nucleotide unit in B has a 2'-deoxy-erythro-pentofuranosyl sugar moiety or a ribo-pentofuranosyl moiety; and
C represents a sequence comprising at least two consecutively located locked nucleotide units, at least one of which is an alpha-L-oxy-LNA unit, and which sequence optionally contains one or more (such as 2, 3, 4 or 5) non-locked nucleotide units (such as deoxyribonucleotide units, ribonucleotide units or derivatives thereof) and/or optionally contains one or more (such as 2, 3, 4 or 5) locked nucleotide units, such as a unit selected from the group consisting of oxy-LNA, thio-LNA, amino-LNA (all in either alpha or beta configuration) and derivatives thereof.

23. A construct according to claim 22, in which the three adjacently located nucleotide sequences are in the following order (5' to 3'):
A-B-C.

24. A construct according to any of claims 22-23, which has the formula (in 5' to 3' order):

A-B-C, where

A, B, and C have the same meaning as defined in any of claims 22-23, and where
A has a length of 2-10 (preferably 2-8) nucleotide units;
B has a length of 1-10 (preferably 5-8) nucleotide units;
C has a length of 2-10 (preferably 2-8) nucleotide units; and the overall lenght of the construct is 8-30 (preferably 10-20) nucleotide units.

25. A construct according to any of claims 22-24, in which A represents a sequence of nucleotide units comprising at least three consecutively located locked nucleotide units, at

least one of said locked nucleotide units being selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof.

26. A construct according to any of claims 22-25, in which C represents a sequence of nucleotide units comprising at least three consecutively located locked nucleotide units, at least one of said locked nucleotide units being selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof.

27. A construct according to any of claims 22-26, in which B represents a sequence of least 2 nucleotide units (such as 3, 4, 5, 6, 7, 8, 9 or 10 units), which sequence in addition to the nucleotide unit(s) having 2'-deoxy-erythro-pentofuranosyl sugar moiety(ies) and/or ribo-pentofuranosyl moiety(ies), comprises nucleotides units which are selected independently from the group consisting of: locked nucleotide units (such as alpha-L-oxy-, -thio-, or -amino- nucleotide units) and derivatives thereof.

28. A construct according to any of claims 22-27, wherein the internucleoside linkages independently are selected from the group consisting of -O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -NR^H-P(O)$_2$-O-, -O-P(O,NR^H)-O-, -O-PO(R")-O-, -O-PO(CH$_3$)-O-, and -O-PO(NHR^N)-O-, where R^H is selected form hydrogen and C$_{1-6}$-alkyl, and R" is selected from C$_{1-6}$-alkyl and phenyl.

29. A construct according to any of claims 22-28, in which B comprises at least one internucleotide linkage which is not a -O-P(O)$_2$-O- linkage, such as a phoshorothioate linkage.

30. A construct according to any of claims 22-29, in which B comprises at least one locked nucleotide unit selected from the group consisting of alpha-L-oxy-LNA and derivatives thereof.

31. A construct according to any of claims 22-30, in which A and C comprises at least one alpha-L-oxy-LNA or alpha-L-thio-LNA unit located adjacent to B.

32. An oligonucleotide which has the formula (in 5' to 3' order):

A-B-C-D, in which

A represents a sequence of locked nucleotide units;

B represents a sequence of non-locked nucleotide units, preferably at least one unit has a 2'-deoxy pentofuranose sugar moiety, in which sequence 1 or 2 nucleotide units optionally are substituted with locked nucleotide units, preferably alpha-L-oxy-LNA;
C represents a sequence of locked nucleotide units; and
D represents a non-locked nucleotide unit or a sequence of non-locked nucleotide units.

33. A construct according to any of claims 32, which has the formula (in 5' to 3' order):

A-B-C-D, where

A, B, and C have the same meaning as defined in claim 32, and where
A has a length of 2-6 (preferably 3-5) nucleotide units;
B has a lenght of 4-12 (preferably 6-10) nucleotide units;
C has a length of 1-5 (preferably 2-4) nucleotide units;
D has a lenght of 1-3 (preferably 1-2) nucleotide units; and the overall length of the construct is 8-26 (preferably 12-21) nucleotide units.

34. A construct according to any of claims 32-33, in which
A has a length of 4 nucleotide units;
B has a length of 7-9, preferably 8, nucleotide units;
C has a length of 3 nucleotide units;
D has a length of 1 nucleotide unit; and the overall length of the construct is 15-17 (preferably 16) nucleotide units.

35. A construct according to any of claims 32-34, in which the locked nucleotide units in A and C are beta-D-oxy-LNA units.

36. A construct according to any of claims 32-35, wherein the internucleoside linkages independently are selected from the group consisting of -O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -NR^H-P(O)$_2$-O-, -O-P(O,NR^H)-O-, -O-PO(R")-O-, -O-PO(CH$_3$)-O-, and -O-PO(NHR^N)-O-, where R^H is selected form hydrogen and C$_{1-6}$-alkyl, and R" is selected from C$_{1-6}$-alkyl and phenyl.

37. A construct according to any of claims 32-36, in which B comprises at least one internucleotide linkage which is not a -O-P(O)$_2$-O- linkage, such as a phoshorothioate linkage.

38. An oligonucleotide construct according to any of claims 32-37, in which

B represents a sequence of nucleotide units that makes the construct able to recruit RNase H when hybridised to a target nucleic acid.

39. An oligonucleotide construct which comprises at least one locked nucleotide unit selected from the group consisting of amino-LNA, thio-LNA (both in either alpha-L or beta-D configuration), alpha-L-oxy-LNA, and derivatives thereof;
wherein at least one of the linkages between the nucleotide units is selected from the group consisting of -O-P(O,S)-O-, -O-P(S)$_2$-O-, -NR^H-P(O)$_2$-O-, -O-P(O,NR^H)-O-, -O-PO(R")-O-, -O-PO(CH$_3$)-O-, and -O-PO(NHR^N)-O-, where R^H is selected form hydrogen and C$_{1-6}$-alkyl, and R" is selected from C$_{1-6}$-alkyl and phenyl.

40. A construct according to any of claims 39, which comprises at least one phoshorothioate internucleoside linkage.

41. A construct according to any of claims 39-40, which comprises a subsequence of nucleotide units, said nucleotide units having 2'-deoxy-erythro-pentofuranosyl sugar moieties.

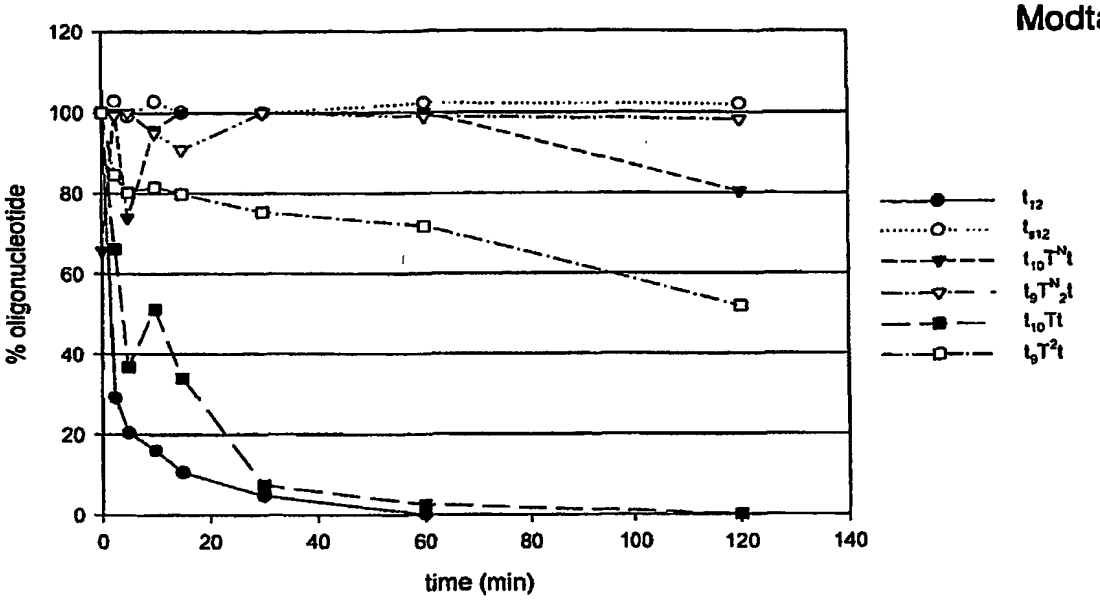


Fig 1

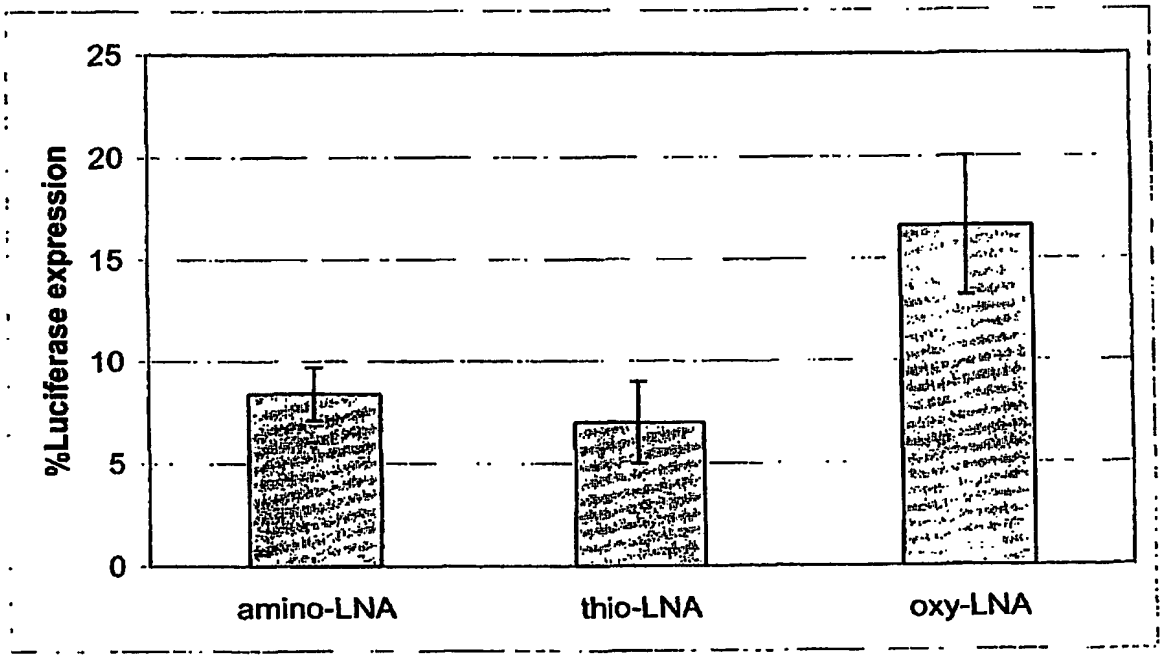


Fig 2

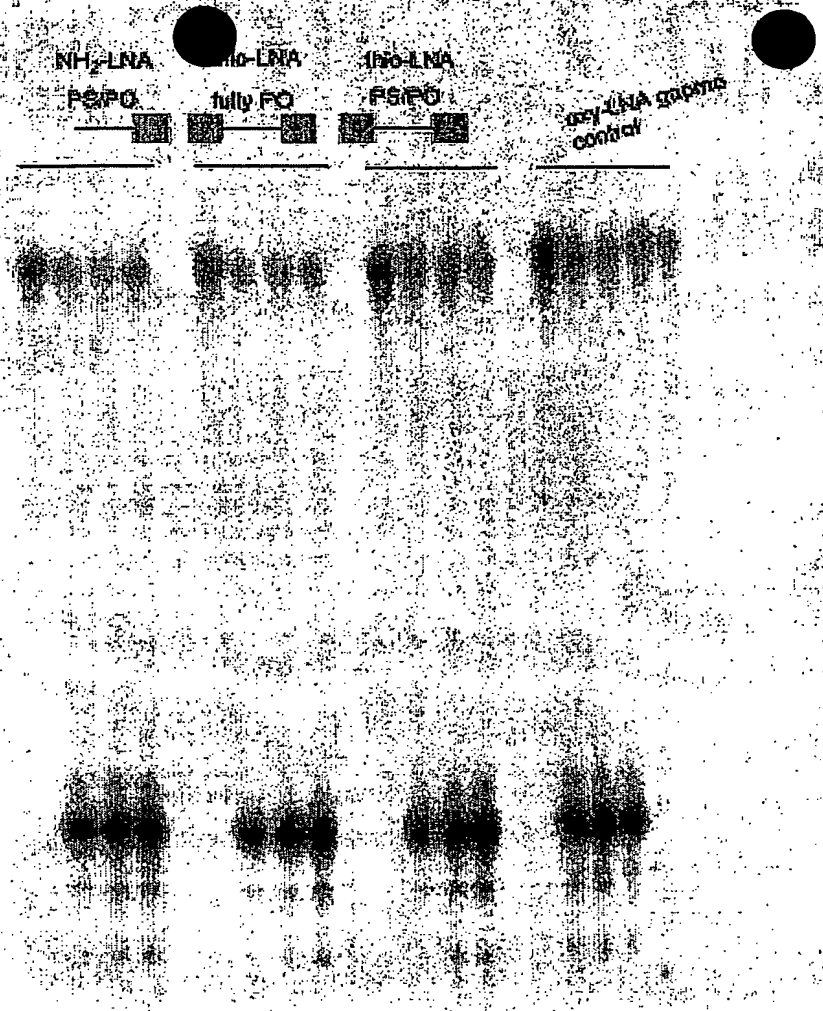


Fig 3

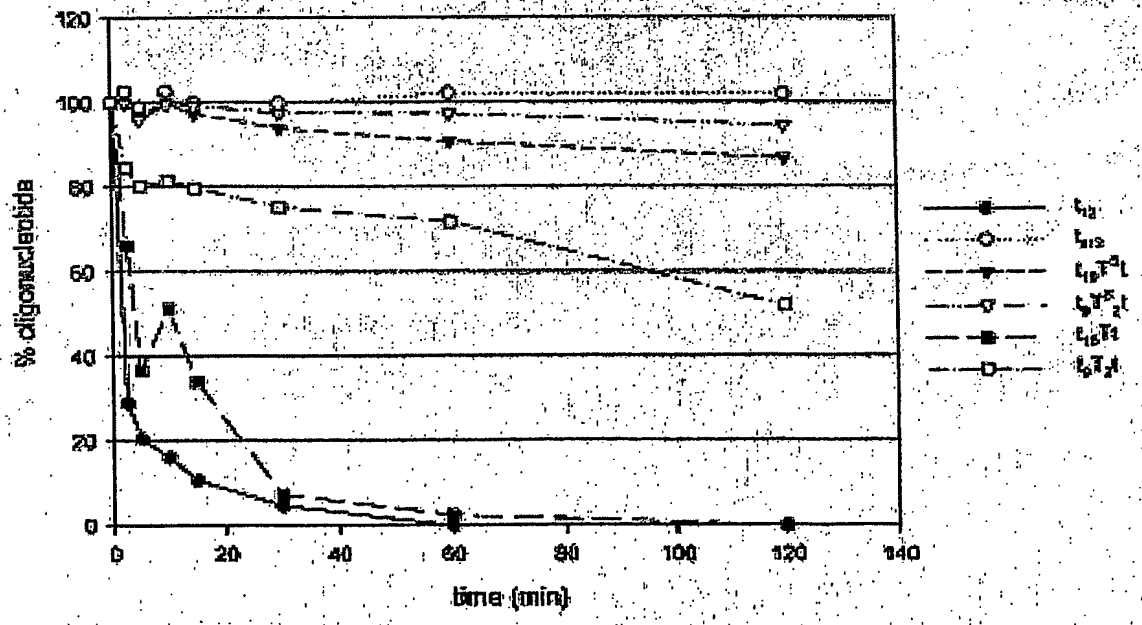


Fig 4

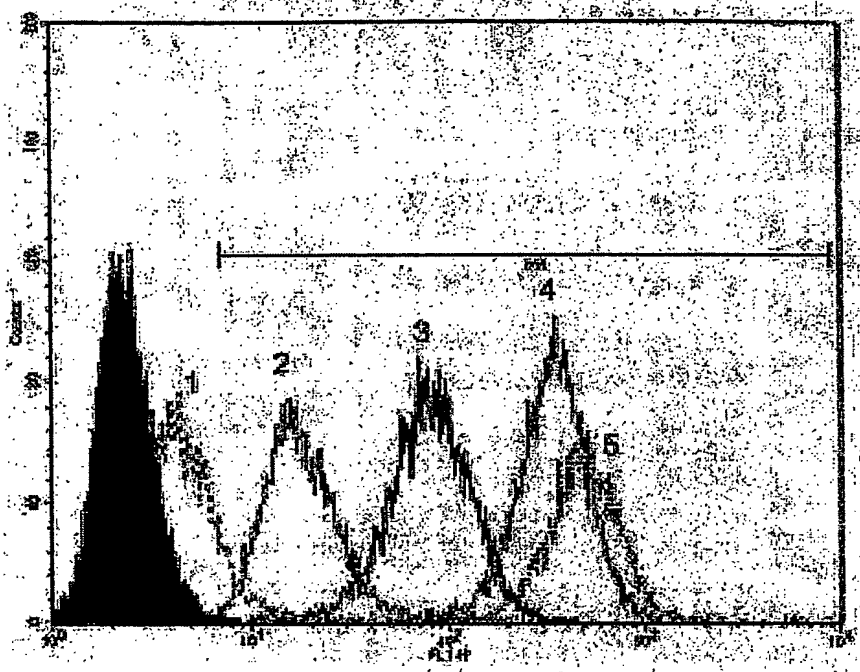


Fig 5

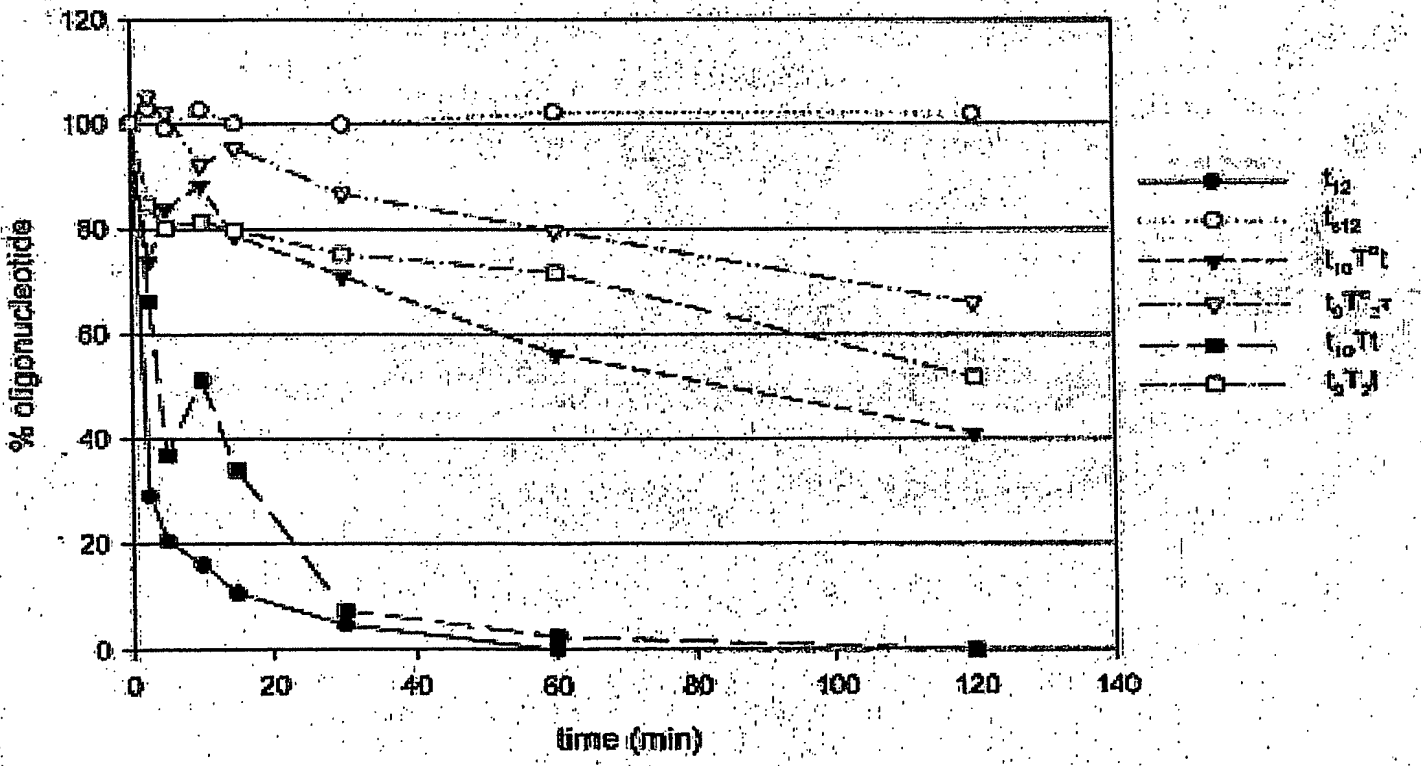


Fig 6

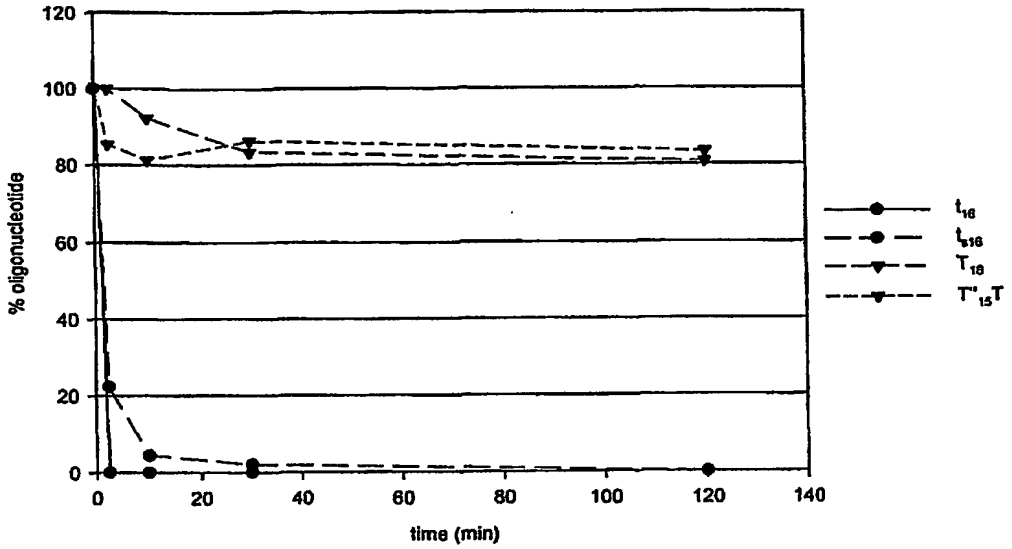


Fig 7

Fig 8

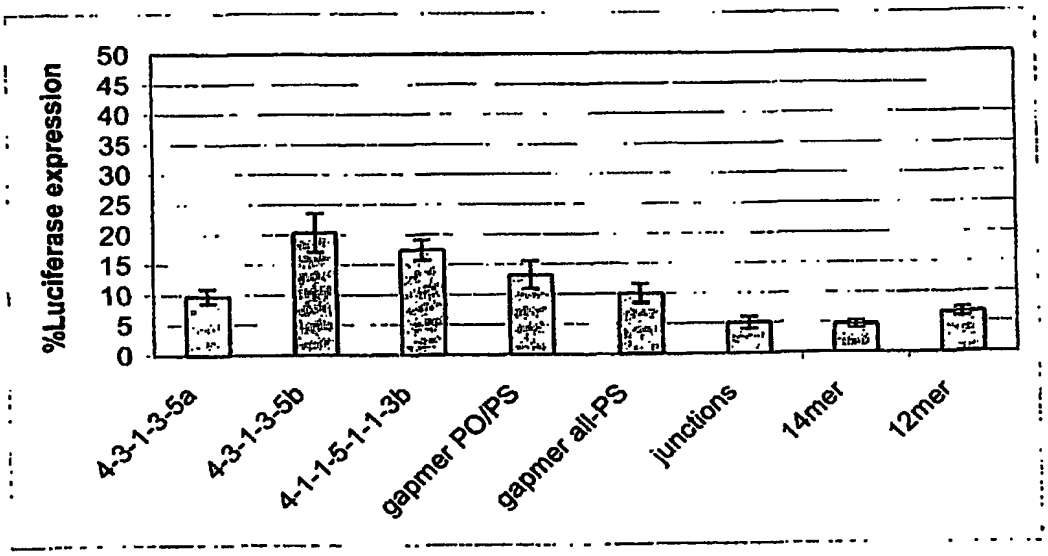


Fig 9

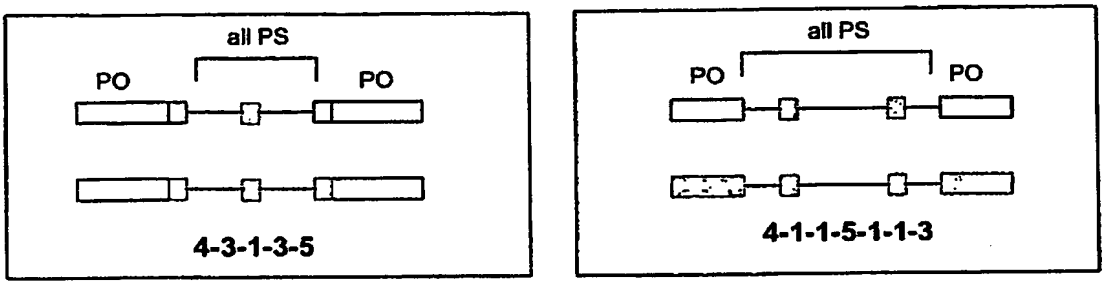


Fig 10

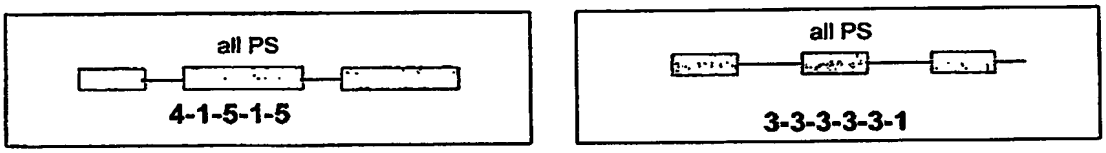


Fig 11

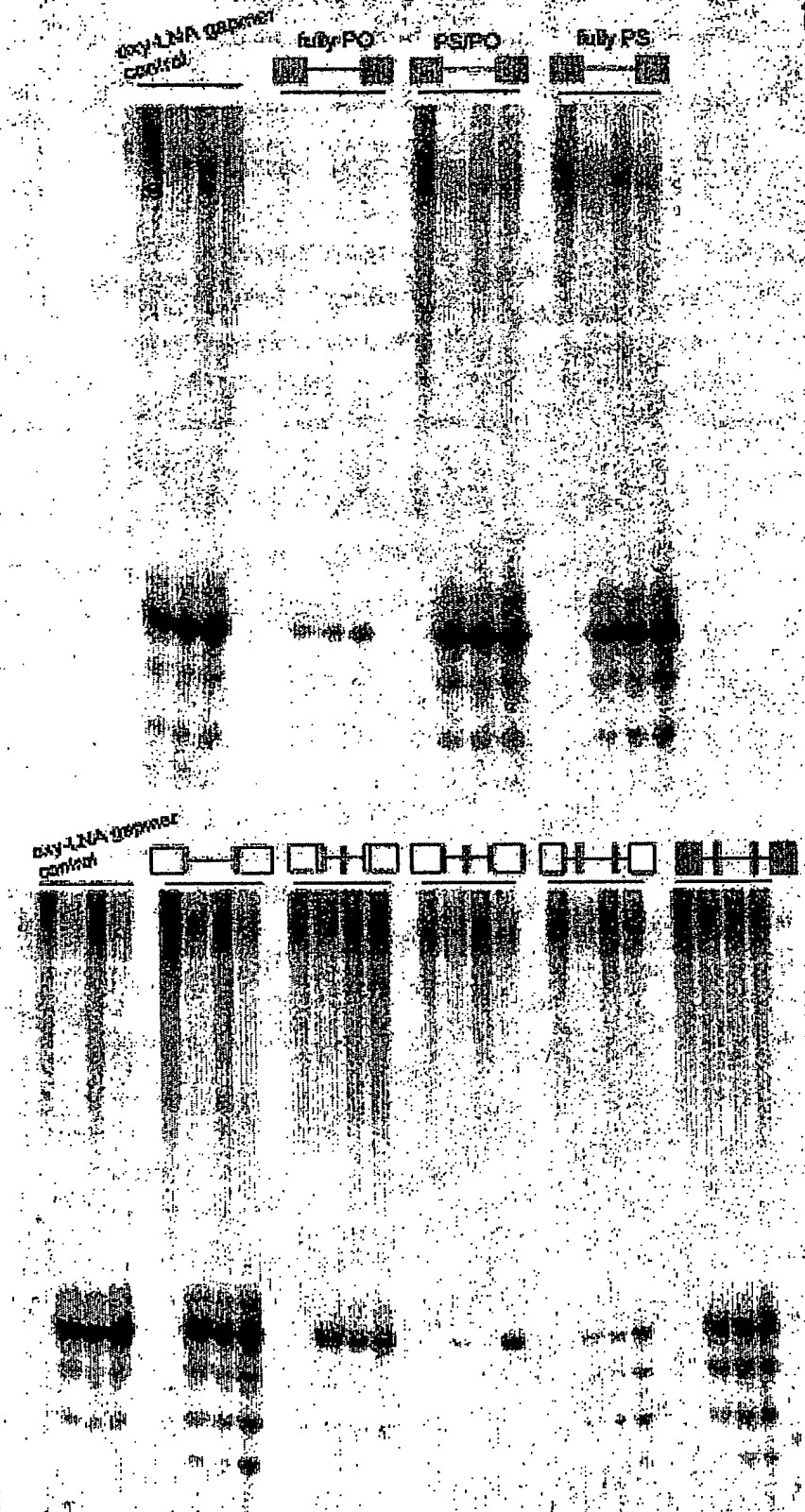


Fig 12

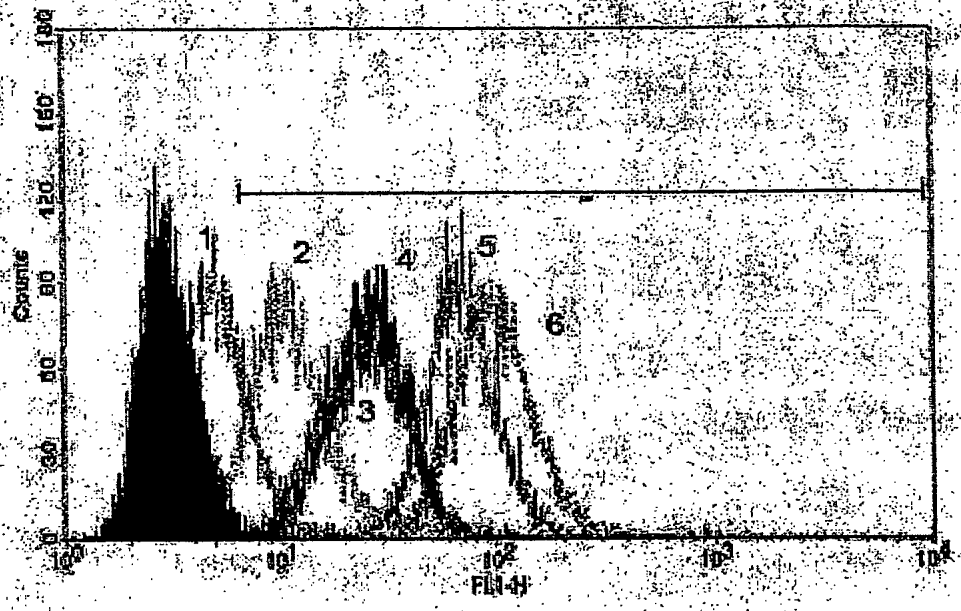


Fig 13

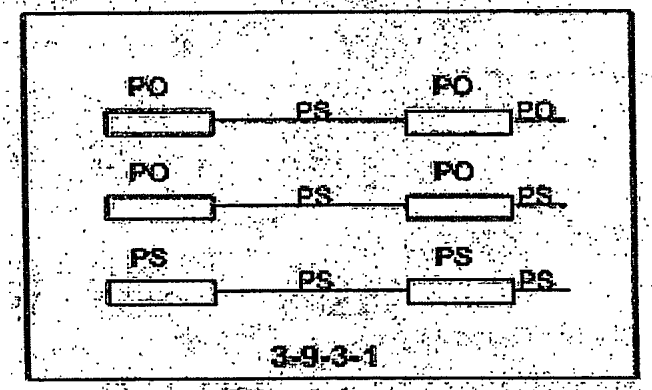


Fig 14

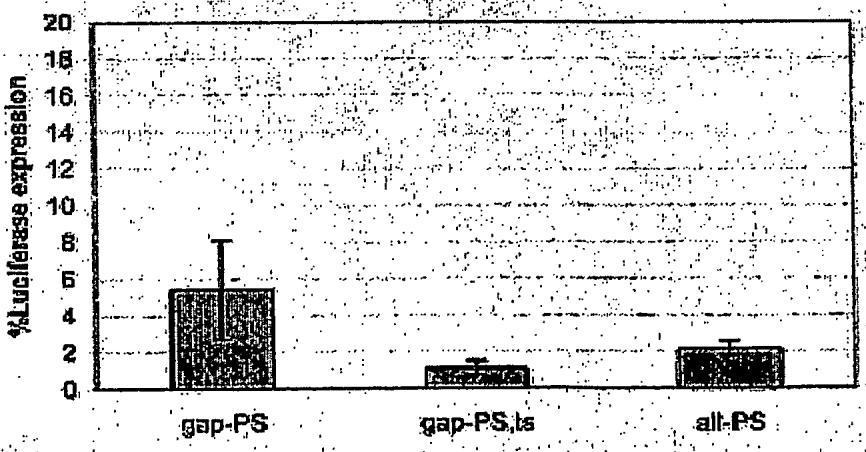


Fig 15